CHARACTERIZATION OF CELLULOLYTIC AND FERMENTATIVE PROCESSES, WITH EMPHASIS ON *Clostridium* SPECIES IN WETLAND SOILS

By

ILKER UZ

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2005

Copyright 2005

by

Ilker Uz

For my parents.

ACKNOWLEDGMENTS

I would like to especially thank Dr Andrew V. Ogram for his excellent advice, strong support and enthusiasm during the course of this dissertation, and for introducing me to world of molecular microbiology. I am thankful to him for the patience he has displayed and financial support that he provided during my study.

I am grateful to Drs. K.R. Reddy, Donald A. Graetz, Kenneth M. Portier, and Joseph P. Prenger for serving on my committee and for their critical review of my research proposal and dissertation.

I want to thank Dr. John Thomas and Bill Reve for their willingness to help me during several stages of my study. I also would like to thank my current and former lab-mates including Dr. Kanika Sharma Inglett, Dr. Hector Castro, Dr. Ashvini Chauhan, Puja Jasrotia, Lisa Stanley, Yannis Ipsilantis, Yun Cheng, and Jason Smith for their friendship, and their assistance during my study. I also acknowledge Dr Yong Ping Duan and Dr Abid Al-Agely.

I wish to thank Turkish Ministry of National Education, and National Science Foundation for their financial support.

Finally, great appreciation is expressed to my family for providing moral support from Turkey, and to my friends for their suggestions when things were difficult.

TABLE OF CONTENTS

page

ACKNOWLEDGMENTS iv

LIST OF TABLES vii

LIST OF FIGURES ix

ABSTRACT xii

1 INTRODUCTION 1

Cellulolytic and Fermantative Systems 2
Genus *Clostridium* 4
Molecular Ecology of *Clostridium* 5
The Florida Everglades 8
Research Hypothesis 10

2 FERMENTATIVE PROCESSES IN WETLAND SOILS 17

Materials and Methods 18
Site Characteristics, Sampling and Biogeochemical Characterization 18
Microbial Enumeration 19
Microcosm Studies 19
Fatty Acid and Methane Measurement 20
Results and Discussion 21
Biogeochemical Characterization 21
Enumeration of Cellulolytic and Fermentative Bacteria 21
Carbon Cycling Potential 22
Effect of Plant Type as Carbon and Nutrient Source on Carbon Cycling 28
Importance of Carbon in Oligotrophic Everglades Soils 31

3 COMPOSITION OF CELLULOLYTIC AND FERMENTATIVE *Clostridium* COMMUNITIES IN THE EVERGLADES SOILS 52

Materials and Methods 53
Site Characteristics, Sampling and Biogeochemical Characterization 53
Soil DNA Extraction 53
Polymerize Chain Reaction (PCR) Amplification 54
Cloning of 16S rRNA Genes and RFLP Analysis 55

Sequencing and Phylogenetic Analysis 55
Terminal Restriction Fragment Length Polymorphism (T-RFLP) Analysis 56
Results and Discussion 57
Phylogenetic Analysis of Cloned *Clostridium* 16S rRNA Gene Sequences 57
Analysis of T-RFLP for *Clostridium* Cluster XIV Species 64

4 CHARACTERIZATION OF FERMENTATIVE PROCESSES IN BENTHIC PERIPHYTON MATS, WITH THE EMPHASIS ON CELLULOLYTIC AND FERMENTATIVE *Clostridium* COMMUNITIES 77

Materials and Methods 78
Site Characteristics, Sampling and Biogeochemical Characterization 78
Microbial Enumeration 78
Microcosm Studies 79
Fatty Acid and Methane Measurement 79
Extraction of DNA and PCR Amplification 80
Cloning of 16S rRNA Genes and RFLP Analysis 80
Sequencing and Phylogenetic Analysis 81
Results and Discussion 81
Biogeochemical Characterization 81
Enumeration of Cellulolytic and Fermentative Bacteria 82
Carbon Cycling Potential 82
Phylogenetic Analysis of *Clostridium* 16S rRNA Gene Sequences from Floc Layer 84

5 SUMMARY AND CONCLUSIONS 98

LIST OF REFERENCES 103

BIOGRAPHICAL SKETCH 111

LIST OF TABLES

Table page

1-1. *Clostridium* species actively hydrolyzing cellulose 13

2-1. Moisture and phosphorus parameters for soil samples from the Everglades in Spring, 2002 33

2-2. Carbon and nitrogen parameters for soil samples from the Everglades in Spring, 2002 33

2-3. Moisture and phosphorus parameters for soil samples from the Blue Cypress Marsh Conservation Area in Fall, 2001 34

2-4. Carbon and nitrogen parameters for soil samples from the Blue Cypress Marsh conservation area in Fall, 2001 34

2-5. Most probable numbers of cellulolytic and fermentative bacteria in Everglades soils 35

2-6. Most probable numbers of cellulolytic and fermentative bacteria in Blue Cypress Marsh soils 35

2-7. Carbon fractionation in various fermentation products in Everglades soil microcosms spiked with glucose 36

2-8. Carbon fractionation in various fermentation products in Everglades soil microcosms spiked with cellulose 36

2-9. Biochemical parameters for plant material used in microcosm experiments 37

2-10. Most probable numbers of cellulolytic and fermentative bacteria in plant microcosms containing soils from F1 and U3 regions of the Everglades 37

3-1. Primers and annealing temperatures used in this study 67

3-2. Result of T-RFLP application for soil samples from the Everglades 68

4-1. Moisture and phosphorus parameters for benthic periphyton samples from the oligotrophic region of the Everglades 88

4-2. Carbon and nitrogen parameters for benthic periphyton samples from the oligotrophic region of the Everglades 88

4-3. Most probable numbers of cellulolytic and fermentative bacteria in benthic periphyton samples 89

4-4. Carbon fractionation in various fermentation products in benthic periphyton microcosms spiked with glucose and cellulose 89

LIST OF FIGURES

Figure page

1-1. Anaerobic degradation of organic matter 14

1-2. Phylogenetic tree of *Clostridium* 15

1-3. Florida Everglades Water Conservation Area 2A 16

2-1. Map of Blue Cypress Marsh Conservation Area 38

2-2. Acetate, butyrate, propionate and methane production potentials of eutrophic and transition soils of the Everglades in microcosms containing no external carbon source 39

2-3. Acetate, butyrate, propionate and methane production potentials of oligotrophic soils of the Everglades in microcosms containing no external carbon source 40

2-4. Effect of glucose on fermentation products and methane production in eutrophic and transition soils of the Everglades 41

2-5. Effect of glucose on fermentation products and methane production in oligotrophic soils of the Everglades 42

2-6. Effect of cellulose on fermentation products and methane production in eutrophic and transition soils of the Everglades 43

2-7. Effect of cellulose on fermentation products and methane production in oligotrophic soils of the Everglades. 44

2-8. Acetate, butyrate, propionate and methane production potentials of high-nutrient and low-nutrient soils of the Blue Cypress Marsh in microcosms containing no external carbon source 45

2-9. Effect of glucose on fermentation products and methane production in high-nutrient and low-nutrient soils of the Blue Cypress Marsh 46

2-10. Effect of cellulose on fermentation products and methane production in high-nutrient and low-nutrient soils of the Blue Cypress Marsh 47

2-11. Effect of plant materials on fermentation products and methane production in Everglades soils 48

2-12. Effect of plant materials on fermentation products and methane production in Everglades soils in the absence of phosphorus in the media 49

2-13. Effect of plant materials on fermentation products and methane production in Blue Cypress Marsh soils 50

2-14. Effect of cellulose, glucose and nitrogen (NH_4-N) additions to microcosm with U3 soils under different level of phosphorus amendments 51

3-1. Rarefaction analysis for *Clostridium* Cluster I clone libraries for soil samples from the Everglades 69

3-2. Phylogenetic tree of *Clostridium* Cluster I 16S rRNA gene clone sequences obtained from Everglades soils 70

3-3. Rarefaction analysis for *Clostridium* Cluster III clone libraries for soil samples from the Everglades 71

3-4. Phylogenetic tree of *Clostridium* Cluster III 16S rRNA gene clone sequences obtained from Everglades soils 72

3-5. Rarefaction analysis for *Clostridium* Cluster IV clone libraries for soil samples from the Everglades. 73

3-6. Phylogenetic tree of *Clostridium* Cluster IV 16S rRNA gene clone sequences obtained from Everglades soil 74

3-7. Rarefaction analysis for *Clostridium* Cluster XIV clone libraries for soil samples from the Everglades 75

3-8. Phylogenetic tree of *Clostridium* Cluster XIV 16S rRNA gene clone sequences obtained from Everglades soils 76

4-1. Acetate, butyrate, propionate and methane production potentials of benthic periphyton in microcosms containing no external carbon source 90

4-2 Effect of different carbon substrates on fermentation products and methane production in benthic periphyton 91

4-3. Rarefaction analysis for Cluster I clone libraries for benthic periphyton samples .. 92

4-4. Phylogenetic tree of *Clostridium* Cluster I 16S rRNA gene clone sequences obtained from benthic periphyton samples 93

4-5. Rarefaction analysis for Cluster III clone libraries for benthic periphyton samples 94

4-6. Phylogenetic tree of *Clostridium* Cluster III 16S rRNA gene clone sequences obtained from benthic periphyton samples 95

4-7. Rarefaction analysis for Cluster IV clone libraries for benthic periphyton samples 96

4-8. Phylogenetic tree of *Clostridium* Cluster IV 16S rRNA gene clone sequences obtained from benthic periphyton samples 97

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

CHARACTERIZATION OF CELLULOLYTIC AND FERMENTATIVE PROCESSES, WITH EMPHASIS ON *Clostridium* SPECIES IN WETLAND SOILS

By

Ilker Uz

May 2005

Chair: Andrew V. Ogram
Major Department: Soil and Water Science

In anaerobic environments such as wetlands soils, microbial processes and microbial community structure are a function of chemical parameters. Two of the major microbial groups present in wetlands are cellulolytic and fermentation species that provide carbon to other groups. It is likely that any biogeochemical changes in these environments may affect these groups, hence affecting the entire microbial food web. This study was conducted with soil and detrital material collected from three different sites in the Everglades Water Conservation Area 2A, characterized by different levels of eutrophication.

Microcosm experiments indicated that carbon cycling mechanisms are more active in eutrophic soils than in oligotrophic soils. Eutrophic and transition soils possess microbial communities or well-established microbial associations that can eliminate excess fatty acids or other by-products. Most probable number (MPN) enumeration of fermentative organisms, and total consumption of glucose and acetate production in the

first 7 days in eutrophic and oligotrophic soils indicated similar activities of fermentative bacteria in the two soils, emphasizing the importance of syntrophic activity (syntrophs and hydrogenotrophic methanogenesis) in fermentation patterns. Plant type as carbon and nutrient source appeared to be significant only in eutrophic soils, based on methanogenesis; and it seemed that sawgrass stimulated carbon cycling. However, MPN fermentative bacteria indicated that cattail has greater impact, possibly because of its higher soluble fraction. Results from microcosms containing different levels of phosphorus and microcosms with plant material suggested that phosphorus might not be limiting for microorganisms in oligotrophic soils.

Culture-independent techniques targeting the 16S rRNA genes of *Clostridium* species revealed significant differences in *Clostridium* assemblage structure based on origin of soil samples. Especially, *Clostridium* Clusters I, IV, and XIV communities appeared to be affected by eutrophication. Possibility of application of T-RFLP method was explored on these soils. Only Cluster XIV was found to be suitable for T-RFLP application to monitor microbial changes in Everglades soils.

Eutrophication clearly affected carbon cycling mechanisms in Everglades soils. Results showed that fatty-acid-consuming bacteria associated with hydrogen-scavenging methanogens play an important role in carbon cycling. This association affects fermentative and cellulolytic organisms. The present study contributed to a greater understanding of carbon cycling mechanism in wetland environments and provided a molecular database that will contribute to monitoring of ecosystem restoration technologies.

CHAPTER 1
INTRODUCTION

Anaerobic degradation of organic matter leading to methane production occurs naturally in many environments such as wetlands, lake sediments, and rice paddies. The process from first to final stage is a result of the activity of microbial consortia. Cellulose (the main carbon source) is degraded by cellulolytic bacteria resulting, in cellobiose and glucose, some of which is used by the same bacterial group to maintain cell activities. The remaining glucose is converted to butyrate, lactate, propionate, acetate, ethanol, CO_2, and H_2 by a secondary bacterial group, fermenters. Then hydrogenotrophic bacteria use H_2 produced in the previous steps to generate acetate and methane from CO_2, H_2S from sulfate, and ammonia and N_2 from nitrate. Acetate is also converted to methane by some methanogens.

There are also secondary fermenters, the syntrophic bacteria, that have a significant role in converting butyrate and propionate to acetate and H_2 (Coughlan and Mayer 1991). All of these groups share the same environments, and the degree and direction of their interactions dictates the conditions. Cellulolytic and fermentative bacteria are two groups that supply carbon to higher trophic groups, such as syntrophs, methanogens and sulfate-reducing bacteria (Garcia et al. 2000) (Figure 1-1). In an anaerobic system, glucose is theoretically converted to methane according to the following reaction:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$$

$$2CH_3COOH \rightarrow 2CH_4 + 2CO_2$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$

Each year, more than 10^{11} tons of dry plant material are produced by photosynthetic fixation of carbon, and approximately half of this material is made of cellulose. Therefore, degradation of cellulose is one of the major and vital processes controlling global carbon cycling. Cellulose is a polymer of glucose monomer units that are oriented 180° to the next. This orientation is the major structural feature that makes cellulose strong and insoluble compared to other polymers of glucose (such as starch, which has glucose molecules oriented in the same direction). Glucose chains form cellulose fibrils in which cellulose molecules are held together by interchain hydrogen bonds and van der Waals interections between pyranose rings (Eriksson et al. 1990; Leschine 1995; Beguin and Lemaire 1996).

Cellulolytic and Fermantative Systems

In general, cellulolytic enzyme systems contain three major enzyme groups: endoglucanase, exoglucanase, and glucosidase. Endoglucanase randomly hydrolyzes 1,4 ß bonds at amorphous sites in the cellulose chain, generating oligosaccharides with different sizes. Exoglucanase cleaves cellobiose or glucose molecules from the oligosaccharide chain. Glucosidase hydrolyzes cellobiose to glucose molecules. Even though the general characteristics are similar, there is a huge difference between aerobic and anaerobic cellulase systems. Unlike aerobic bacteria, anaerobic bacteria generally have localized cellulose components on their cell surface and optimum cellulose use occurs when cells are attached to substrate. This maximizes efficient penetration to cellulose material and uptake of cellobiose and glucose to the host cell without competition from noncellulolytic fermentation bacteria. Related to that, anaerobic bacteria, with one exception, form high-molecular-weight complexes called cellulosomes. For example, *Clostridium thermocellum* (the most-studied anaerobic

cellulolytic bacteria) has 22 catalytic modules in their cellulosomes. At least 9 function as endoglucanases, 4 exhibit exoglucanase activities, and 5 show hemicellulose activities (Coughlan and Mayer 1991; Leschine 1995; Beguin and Lemaire 1996; Schwarz 2001; Lynd et al. 2002). Only *Clostridium stercorarium* does not appear to have the multi-enzyme complex (Table 1-1) (Schwarz 2001).

Expression of cellulosome genes is controlled by the end-products cellobiose and glucose. In the environment, however, fermenters have direct control on cellulolytic activity by keeping glucose and cellubiose concentrations at low levels by using excess amounts. High levels of glucose or more reduced substrates appear to promote production of butyrate, lactate, and propionate, instead of acetate, since these products act as hydrogen sinks (Jones and Woods 1986; Pryde et al. 2002). NADH oxidoreductase and hydrogenase play an important role in controlling electron and carbon flow in some fermenters. Normally, acetate formation from glucose is the most favorable route, since this provides the maximum ATP and NADH production. However, no NADH is consumed during the process.

The NADH oxidoreductase and hydrogenase enzyme system provides the NADH recycling service; and the end product, H_2, is released to the environment without consuming any carbon compound. However, this reaction occurs only when pH value is above 6.8 (Jones and Woods 1986). If hydrogen consumption in anaerobic systems by hydrogenotrophs is not sufficient, fermenters produce less oxidized products, such as lactate, ethanol, butyrate, and propionate. As a result, fermenters, through their end products, have some level of control on microbial community composition in higher trophic levels, especially on sythrophs. However, it is also true that fermenters are

affected by the same higher trophic organisms (Miller and Wolin 1995). These interactions and benefits also depend on the organisms involved. Cellulolytic activity may be directly or indirectly affected considering the fact that cellulolytic bacteria are also fermenters. In a study with cellulolytic strains (18P13) and two H_2-utilizing methanogens or acetogens (*Methanobrevibacter smithii* and *Ruminococcus hydrogenotrophicus*) isolated from the human colon, the *M. smithii*-18P13 combination resulted in a metabolic shift in 13P13 with no difference in cellulolytic activity. However, *R. hydrogenotrophicus* increased the cellulose breakdown by strain 18P13 (Robert et al. 2001).

Genus *Clostridium*

Clostridium is a large genus of gram-positive, endospore-forming, anaerobic bacteria. Their anaerobic metabolic activities lead to fatty acids and other organic products that a have strong odor. Even though most *Clostridia* are aerotolerant, *Clostridium* species vary significantly in their oxygen tolerance. Most anaerobic cellulolytic bacteria identified belong to genus *Clostridium* and its relatives (Schwarz 2001). This group also contains many noncellulolytic fermentation bacteria. Analysis of the 16S rRNA gene sequence revealed that the *Clostridium* group is highly diverse and is divided into 19 subclusters (Figure 1-2) (Collins et al. 1994). Celluloytic *Clostridium* species are located in a number of different clusters such as cluster I, III, IV and XIVab. However, cluster III is the only group that consists solely of cellulolytic bacteria (Collins et al. 1994; Van Dyke and McCarthy 2002).

Cluster I is the largest *Clostridium* cluster and contains the type species of the genus, *Clostridium butyricum*, and half of the pathogenic *Clostridium* species. Therefore, it is considered to be the core cluster. Cluster I includes species producing solvents such

as butanol, and a variety of fatty acids (such as acetate, butyrate, and lactate), and some of them (such as *C. cellulovorans*) have the ability to degrade cellulose.

Cluster II is made of three species whose sequences are similar to each other with >96%. These species also show similar acetogenic and proteolytic capability. Members of this cluster are less than 92% similar to cluster I species. However, it is problematic to separate cluster II from the cluster I with respect to phenotypic characteristics.

Cluster III consists of both mesophilic and thermophilic cellulose-degrading *Clostridium* species. Sequence similarity of species to each other ranges from 87 to 99%.

Cluster IV contains species phenotypically heterogeneous, and G-C contents of chromosomes vary broadly. Cluster IV may present a suprageneric or family group. Members of this cluster include representatives from genera *Clostridium*, *Eubacteria*, and *Ruminococcus*; some of which can utilize cellulose, such as *C. cellulose*, *R. albus*, and *R. flavefaciens* (Van Dyke and McCarthy 2002).

Cluster XIV contains different clostridial species with sequence similarity of 80-99% and generally higher G-C content. Members of Cluster XIV include cellulolytic and noncellulolytic species from human, animal gut, rumen, and environmental sources. Cluster XIV is divided to two distinct subclusters. Cluster XIVa consists of phenotypically heterogeneous organisms, some of which are nonspore-forming cocci (e.g. *Ruminococci*). Evolutionary distances and heterogeneous diversity in phenotypical features suggest that cluster XIVa is a suprageneric cluster. Cluster XIVb is made of species with loose association, and three branches exist (Collins et al. 1994).

Molecular Ecology of *Clostridium*

Most information available about the *Clostridium* group and their function in the environment comes from culture-based techniques and pure culture studies. Due to the

fact that conventional cultivation techniques have limitations in assessing the diversity of naturally occurring microbial communities, new methods are needed. Molecular techniques such as phylogenetic analysis based on rRNA gene amplification and DNA sequencing provide great opportunity to scientists working in this area.

Surprisingly, microbial diversity and molecular ecology studies of *Clostridium* species in the environment have received little attention to date. Only recently, a limited number of studies involving molecular techniques on this group in various environments, such as landfills, and human and animal intestines have been reported. Specific rRNA gene primers targeting various *Clostridium* clusters proved to be reliable tools. These studies indicated the widespread presence of cluster III and IV *Clostridium*, but not cluster I, in landfill samples (Van Dyke and McCarthy 2002). Considering the lack of studies on ecology of *Clostridium*, this provides valuable information. Another molecular study focusing on general bacterial population involving rice straw decomposition in anoxic paddy soil showed the importance of Clostridial species. A bacterial community degrading rice straw established in the first 15 days dominated by *Clostridium* clusters I, III, and XIVa (Weber et al. 2001). Clostridial species were also detected in high-alkaline anaerobic environments (Borsodi et al. 2003). Molecular techniques such as Fluorescent In Situ Hybridization (FISH) have been reported recently to enumerate this group. A study conducted on human fecal samples using FISH found a low number of cluster I-II *Clostridium*. However, probes targeting cluster XIVab counted 29% of flora in these samples (Franks et al. 1998). Similarly, in a methanogenic landfill leachate bioreactor, 100% of the library from cellulose-attached fraction and 90% of the library from mixed fraction (organisms attached to cellulose molecules and in the planktonic phase) were

found to be *Clostridium* species. That study detected a possible new cluster designated as cluster VIa (Burrell et al. 2004). Numberous studies also used more-specific PCR primers targeting a limited number of cellulolytic species (such as *Fibrobacter succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefaciens*) in rumen or animal intestines. These primers detected previously undiscovered *Fibrobacter succinogenes*-like subspecies in the gastrointestinal content of a pony (Lin and Stahl 1995); and detected more *F. succinogenes* than other cellulolytic bacteria in sheep rumen (Koike and Kobayashi 2001).

Even though phylogenetic analysis provides valuable information about community structure, it is time-consuming and costly. If the purpose is to monitor changes in diversity and community structure in response to chemical or physiological changes, the technique to be used should be fast and inexpensive. It should also allow analysis of a large number of samples at the same time. Denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and single-strand conformation polymorphism (SSCP) provide these advantages (Osborn et al. 2000). An additional solution to these problems is terminal-restriction fragment length polymorphism (T-RFLP). This technique is based on PCR with primers that are labeled with a fluorescent dye. After amplification, PCR products are digested with an enzyme, and the size and fluorescence intensity of the labeled fragment is measured by electrophoresis equipped with a laser detector. This method lets scientists compare microbial diversity and changes in microbial structure in different environmental samples. The major disadvantage of this method is that the result obtained cannot be

evaluated quantitatively because of primer bias (Osborn et al. 2000). To our knowledge, the T-RFLP method has not been used for *Clostridium* communities in environment.

The Florida Everglades

The Florida Everglades, historically a low-nutrient system, is one of the anaerobic environments in which all of the interactions mentioned above are present. Moreover, it has been suffering from nutrient loading associated with nearby agricultural activities. Phosphorus loading is the major contributor of changes to plant and microbial ecology (Wright and Reddy 2001b). Thus, eutrophic regions are now dominated by cattail (*Typha* sp.) and large amounts of nutrient-enriched organic matter; and pristine regions are dominated by sawgrass (*Cladium* sp.) and lower accumulations of nutrient-poor organic matter (Figure 1-3). Analysis and simulations for vegetation pattern changes in the Everglades show that sawgrass will be replaced by cattail when the soil total phosphorus level exceeds 650 mg/kg (Wu et al. 1997). Organic matter accumulation is a result of imbalance between inputs and outputs. In the Everglades, the vertical peat accumulation rate was found to be between 1.1 cm g^{-1} and 0.25 cm g^{-1} along the phosphorus gradient; and the carbon mineralization rate was found to be related to phosphorus concentration. Phosphorus accumulation rates were between 0.54 and 1.14 g P yr^{-1} in impacted sites, and 0.11 and 0.25 g P yr^{-1} in unimpacted sites (Reddy et al. 1993).

It is reported that 91% of variability in aerobic decomposition is due to phosphorus and lignocellulose composition. Anaerobic carbon turnover was one-third of aerobic rates (DeBusk and Reddy 1998). This indicates that, in the Everglades, carbon turnover is regulated by phosphorus and substrate quality. A study conducted with stable carbon isotopes showed that most of the dissolved organic carbon (DOC) in the Florida Everglades Water Conservation Area 2A (WCA-2A) is derived from historic peat

deposits. However, pristine areas showed a younger radiocarbon signature. Therefore, it was suggested that the radiocarbon signature of DOC can be used as indicator for restoration efforts (Wang et al. 2002). Entry (2000) indicated that C:N ratio of organic matter may not be a good predictor of decomposition because of differences in carbon quality of the substrate. Instead, the cellulose:lignin:N ratio should be used to predict the decomposition rate in wetlands. Seasonal changes in temperature, water level, and oxidation-reduction regime results in significant differences in eutrophic regions of the Everglades. However, possibly because of phosphorus and organic substrate limitations, pristine regions show no significant difference in microbial processes (Kochrose et al. 1994). Newman et al. (2001) indicated that phosphorus loading impacts the decomposition rate in mesocosms, and that the cotton rottenness rate (CRR) can be used as an indicator of microbial response. Other researchers studied alternative methods (such as extra cellular enzyme assays) to assess biological activities.

Although phosphorus loading and decomposition rates are clearly related, a similar relationship could not be observed with ß-glucosidase activity measured in anaerobic soil in the Everglades. However, CO_2 production and ß-glucosidase activity correlated significantly in drained detritus and soil (Wright and Reddy 2001a, 2001b). Freeman et al. (1996) observed increased activity of ß-glucosidase without any increase in microbial respiration when the water table was lowered in Welsh peatland. They attributed the increased enzyme activity to reduction of inhibitory effect of phenolic compounds and iron on extracellullar enzymes already present in the system. Later, it was proposed that the low decomposition rate in peatlands is due to inhibition of a single enzyme, phenol oxidase, because of oxygen limitation. Phenol oxidase is one of the rare enzymes that can

degrade the phenolic compounds that inhibit hydrolytic enzymes in the environment (Freeman et al. 2001). Oxygen limitation, however, may not be the only reason for inhibition of hydrolytic enzymes. High-level anthropogenic nitrate deposition may also suppress these enzymes (DeForest et al. 2004).

Concurrent studies have shown that eutrophication in wetlands changes the composition and activities of lower trophic groups. Impacts of nutrients on processes such as methanogenesis and respiration have been well studied; however, few studies have focused on the impacts of nutrients on the composition and activities of microorganisms responsible for these processes. A rare report on the microbial ecology of the Everglades indicated 10^3 to 10^4 times higher numbers of anaerobic microorganisms in eutrophic soil samples than in pristine soil samples. It was concluded that anaerobic microflora in nutrient-impacted soils respond faster to phosphorus, sulfate, and nitrate input (Drake et al. 1996). More recent studies reported a metabolic selection among sulfate-reducing bacteria (Castro et al. 2002) and methanogenic and syntrophic bacteria (Chauhan et al. 2004) according to nutritional status of the Florida Everglades soils.

Research Hypothesis

In nature, cellulose is generally present with other compounds such as lignin, hemicellulose, pectin, and proteins. Interactions among these components make cellulose degradation a more complicated and valuable area of research. However, this area has remained largely unexplored due to the inherent problems associated with the complex degradation pathways involved in the breakdown of cellulose and fermentation in the environment. This is especially true in the Florida Everglades because it has been suffering from nutrient loading due to agricultural activities. Even though a few studies have examined lower pathways of carbon cycling in the Everglades, cellulose

degradation and fermentation must also be investigated to understand the true microbial nature of this site and the impact of nutrient loading on carbon cycling mechanisms.

Our overall goal was to draw a more complete picture of nutrient impacts on carbon cycling, including establishing linkages between carbon input and microbial community structure and function. We hypothesize that the composition and metabolism of cellulolytic and fermentative *Clostridium* group is a function of the nutritional status of the Everglade soils. Specific hypotheses were proposed to gain information about cellulose degradation and fermentation in the Florida Everglades.

- **Hypothesis 1:** Accumulation of nutrient rich organic material in nutrient impacted sites correlates with relatively larger population size in cellulolytic community.
- **Hypothesis 2:** Nutritional status of soils correlates with the composition of cellulolytic and fermentative species.
- **Hypothesis 3:** Impacted soils contain a microbial community that is poised to respond more rapidly to changes in nutritional status compared with nonimpacted soils.

Study Overview

Our study was conducted to understand the terminal carbon cycle, specifically cellulose degradation and fermentation, in anaerobic environments; and relationships among the organisms responsible for these processes. We also aimed to investigate the response of these organisms to nutrient loading observed in the Florida Everglades soils. Soil samples were collected from nutrient impacted, transient, and nonimpacted regions of the Everglades Water Conservation Areas 2A (WCA-2A).

Chapter 2 focuses on the characterization of fermentation processes and fermentation product patterns under different carbon sources. This chapter also investigates the importance of plant type as a nutrient source for fermentation process.

Experiments described in this chapter also include soils samples from Blue Cypress Marsh to observe whether the results from the Everglades are unique to this region.

Chapter 3 investigates and compares the composition of cellulolytic and fermentative organisms, specifically *Clostridium* species, with molecular techniques targeting 16S rRNA gene. In addition, possibility of application T-RFLP (Terminal Restriction Fragment Length Polymorphism) method for *Clostridium* species on the Everglades soils was explored.

Chapter 4 describes cellulolytic and fermentative communities and their functions in benthic periphyton in nonimpacted region with combination of molecular techniques and analytical methods. Results obtained from benthic periphyton samples were compared with those from soils samples.

Finally, Chapter 5 summarizes implications of this research toward greater understanding of terminal carbon mineralization, and factors controlling this process in Everglades wetland soils.

Table 1-1. *Clostridium* species actively hydrolyzing cellulose

Organism	Optimum growth temperature	Source	Evidence for production of cellulosomes[a]
Clostridium acetobutylicum	Mesophilic	Soil	(+)[b]
Clostridium chartatabidum	Mesophilic	Rumen	
Clostridium cellulovorans	Mesophilic	Wood fermenter	+
Clostridium herbivorans	Mesophilic	Pig intestine	
Clostridium cellulosi	Thermophilic	Manure	
Clostridium cellobioparum	Mesophilic	Rumen	+
Clostridium papyrosolvens	Mesophilic	Paper mill	+
Clostridium josui	Thermophilic	Compost	+
Clostridium cellulolyticum	Mesophilic	Compost	+
Clostridium aldrichii	Mesophilic	Wood fermenter	
Clostridium stercorarium	Thermophilic	Compost	-
Clostridium thermocellum	Thermophilic	Sewage soil	+
Clostridium cellulofermentans	Mesophilic	Manure	
Clostridium celerescens	Mesophilic	Manure	
Clostridium thermopapyrolyticum	Thermophilic	Mud	
Clostridium thermocopriae	Thermophilic	Hot spring	
Clostridium sp. C7	Mesophilic	Mud	+
Bacteroides sp. P-1	Thermophilic	Rotting biomass	+
Bacteroides cellulosolvens	Mesophilic	Sewage	+
Acetivibrio cellulolyticus	Mesophilic	Sewage	+
Acetivibrio cellulosolvens	Mesophilic	Sewage	

Adapted from Schwarz (2001).

[a] Evidence for production of cellulosomes refers to observations on molecular, biochemical and microscopic properties of organisms

[b] Even though a complete cellulosomal gene cluster is present in its genomic DNA, no cellulolytic activity has ever been reported for *Clostridium acetobutylicum*.

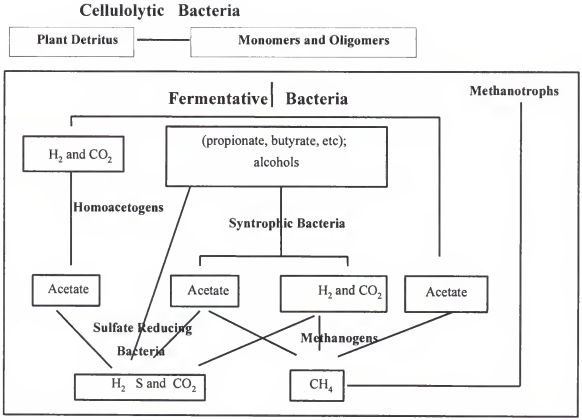

Figure 1-1. Anaerobic degradation of organic matter

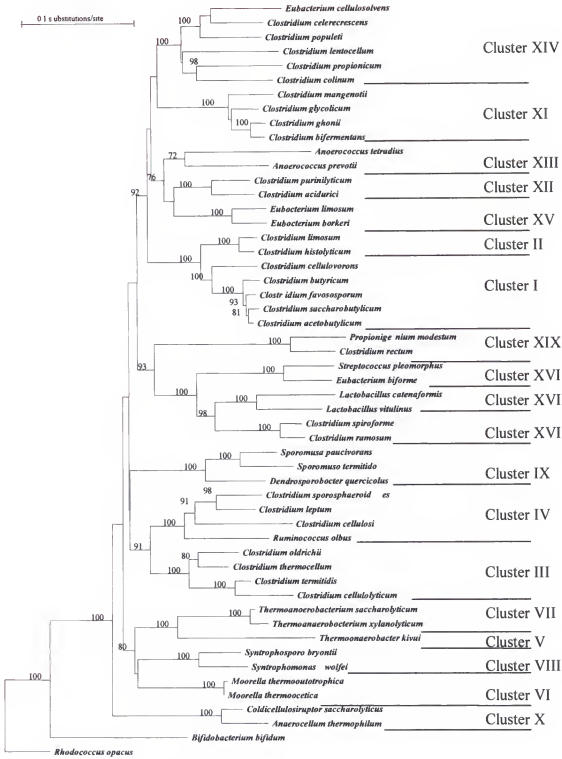

Figure 1-2. Phylogenetic tree of *Clostridium*

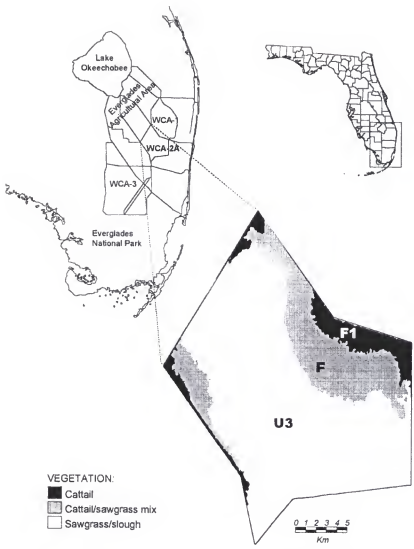

Figure 1-3. Florida Everglades Water Conservation Area 2A (WCA-2A)

CHAPTER 2
FERMENTATIVE PROCESSES IN WETLAND SOILS

Cellulose and its degradation products are among the most important carbon sources for microbial groups present in natural anaerobic environments. Therefore, cellulolytic and fermentative organisms initiate much of the turnover of carbon in these environments. Despite their importance, these processes have not been characterized in detail for Everglades soils. Microbial studies published on the Everglades, in general, focus on functions of other groups that are dependent on decomposition of cellulose, such as homoacetogenesis (Drake et al. 1996), methanogenesis and syntrophy (Chauhan et al. 2004; Castro et al. 2004), and sulfate reduction (Castro et al. 2002). These studies did not address relationships between various members of these food webs, or the importance of cellulolytic and fermentative organisms as possible driving forces in carbon cycling.

We investigated potential fermentation of various carbon sources (glucose, cellulose and plant material) in Everglades soils, to compare nutrient loading and effect of type of plant material on carbon mineralization. Microcosm experiments provided information on relationships among different bacterial groups (fermentative, syntrophic, and methanogenic organisms), since production and consumption patterns of known fatty acids and methane production rates were measured. Results obtained from Everglades soils were compared with results from Blue Cypress Marsh soils, which have a similar history in terms of nutrient impact.

Materials and Methods

Site Characteristics, Sampling and Biogeochemical Characterization

Soil samples were collected by South Florida Water Management District staff from eutrophic, transition, and oligotrophic regions of the Florida Everglades Water Conservation Area 2A (WCA2A) under flooded conditions in Spring, 2002. Eutrophic regions (F1) are dominated by cattail [*Typha domingensis*], and transition regions (F4) are dominated by mixture of cattail and sawgrass [*Cladium jamaicense*]. Oligotrophic regions (U3) are dominated by only sawgrass. Soil cores were collected in triplicate and transferred overnight to the Wetland Biogeochemistry Laboratory at the Soil and Water Science Department (University of Florida, Gainesville). After removal of the detrital layer, cores were sectioned and soils corresponding to 0 to 10 cm depth were separated for further analysis. Replicate soil samples were manually mixed and composite subsamples were prepared for storage. Subsamples to be used for microcosm experiments and enumeration were stored at 4°C until analysis (within 2 to 7 days after sampling). Total nitrogen, extractable ammonium-N, total phosphorus, total inorganic phosphorus, extractable organic carbon, and microbial biomass carbon were determined by the Wetland Biogeochemistry Laboratory as described previously (Wright and Reddy 2001a; Castro et al. 2002; Chauhan et al. 2004).

Soil samples from Blue Cypress Marsh (BCM) were also collected. BCM is a fresh-water marsh located in east-central Florida, with a history of nutrient impact from agricultural areas between the 1960s and 1990s. Nutrient loading during the 30-year period resulted in long-lasting changes in the BCM environment, which can still be observed. Northeast (NE) and southwest (SW) regions of BCM are considered eutrophic and are dominated by cattail [*Typha* spp.], and the northwest (BCT) area is the relatively

low-nutrient reference site occupied by sawgrass [*Cladium* spp.] and maidencane [*Panicum* spp.] (Prenger and Reddy 2004). We used soil samples collected from the northeast (eutrophic) and northwest (low nutrient) regions of Blue Cypress Marsh Conservation Area (BCMCA) in Fall 2001 (Figure 2-1). Triplicate soil cores were collected, the top detritus layer was removed, and soil core sections representing 0 to 10 cm depth were mixed to create composite samples. This sample was stored at 4°C until analysis (within 2 to 7 days after sampling). Biogeochemical analysis was conducted as described above.

Microbial Enumeration

The most probable number (MPN) technique with 5 replicates per dilution was used for enumeration studies. The MPN medium contained peptone (10 g/L), NaCl (5 g/L) and bromocresol purple (0.0085 g/L), cysteine-sodium sulfide (2%, to provide final redox potential of –110 to –200 mV). Glucose (20 mM) and cellulose powder were added to MPN tubes for fermentation bacteria and cellulolytic bacteria, respectively. For fermentative MPN, any color change from purple to yellow due to acidity was counted as positive. For cellulose MPN, tubes changing color due to acidity and showing structural change in cellulose substrate were counted positive.

Microcosm Studies

Composite soil samples (2 g, wet weight) were mixed with 50 mL basal carbonate yeast extract trypticase (BCYT) media (Touzel and Albagnac 1983) in 100 mL serum tubes. In each microcosm experiment, 3 or 5 replicates were used for each composite sample from F1, F4, and U3 regions. BCYT also included resazurin (1%), cysteine-sodium sulfide (2%), and a carbon source. Compounds used as carbon source include glucose (20 mM), cellulose (0.162 g, 6.24 mmole C), dried and crushed dead standing

cattail, and sawgrass plants (0.5 g). All media, stock solutions, and microcosms were prepared under nitrogen gas stream to provide anaerobic conditions. For microcosm experiments with plant material, 2 separate sets of microcosms were prepared. One of these sets did not contain phosphorus in the medium. Vials were closed with robber stoppers and aluminum seals, and incubated at 28°C.

Microcosms used in the phosphorus experiment contained 20 g wet soil from the U3 region and 30 mL sterile deionized water. Plant residues in soil were separated and cut into small pieces and equally distributed among microcosms. Phosphorus concentrations tested were 0, 50, 100, 300, 600, 900, and 1200 ppb (0, 2.5, 7.5, 15, 22.5 and 30 mg/kg dry soil). Methane production was monitored for the duration of the experiment. Approximately 2 months after the experiment started, some of the microcosms received cellulose (0.1%), glucose (10 mM), or nitrogen (1 g/L NH_4Cl).

Fatty Acid and Methane Measurement

Liquid samples (1 mL) were collected weekly from microcosms. These samples were centrifuged, filtered through 0.2 µm filters, and stored at –20°C until analysis. Fatty acids were measured with a high-pressure liquid chromatograph (HPLC) (Waters Corp., Milford, MA) equipped with a UV detector set at 210 nm. Aminex HP 87 H column (300 x 7.5 mm) was used with sulfuric acid (0.5 mM) as mobile phase at the flow rate of 0.6 mL/min. Methane formation in the headspace was determined by a Shimadzu 8A gas chromatograph equipped with a Carboxen 1000 column (Supelco, Bellefonte, PA) and flame ionization detector set at 110°C. Nitrogen was used as carrier gas and the oven temperature was 160°C. The pressure in the headspace was measured with a digital pressure device (DPI 705; Druck, New Fairfield, CT).

Results and Discussion

Biogeochemical Characterization

Biogeochemical parameters for phosphorus, carbon and nitrogen in the Everglades and Blue Cypress Marsh soils are shown in Tables 2-1, 2-2, 2-3, and 2-4. In the Everglades soils, total phosphorus and total inorganic phosphorus were found to be higher in eutrophic zones (F1), followed by transition (F4) and oligotrophic (U3) zones (Table 2-1). Similarly, total carbon was found to be higher in F1 soils followed by F4 and U3 soils. However, U3 (ridge) soil has slightly higher total carbon content than F1 soil. Extractable organic carbon was similar in F1 and F4 soils and higher than U3 soil, and lower than U3 (ridge) soil. However, microbial biomass carbon was found to be higher in F4 soils followed by F1. U3 and U3 (ridge) soils have the lowest microbial biomass carbon value. Total nitrogen was slightly higher in U3 and U3 (ridge) soils (Table 2-2). These data are in agreement with previously published reports (Wright and Reddy 2001a, 2001b; Castro et al. 2002, 2004; Chauhan et al. 2004).

In Blue Cypress Marsh, total phosphorus, total inorganic phosphorus, total and extractable total organic carbon, and microbial biomass carbon were found to be higher in eutrophic soils (NE) than in reference soils (BCT). Nitrogen parameters, however, were slightly higher in BCT soils than in NE soils (Tables 2-3 and 2-4).

Enumeration of Cellulolytic and Fermentative Bacteria

In the Everglades, cellulolytic most probable numbers (MPN) were 10 fold higher in F1, F4 and U3 (ridge) than in U3. On the other hand, MPNs of fermentation bacteria were found to be at least 10 times higher in U3 (ridge) soils. No significant differences in MPNs of fermentation bacteria were observed between F1, F4 and U3 soils of Everglades (Table 2-5).

In Blue Cypress Marsh, impacted soils appeared to have 10 times higher cellulolytic microorganisms than did reference soils. However, no significant difference was observed between MPN in impacted and reference soil (Table 2-6).

Carbon Cycling Potential

Results of control microcosms with no external carbon source indicates indigenous carbon cycling potential of soils under the conditions provided. As seen in Figure 2-2 and Figure 2-3, highest acetate and methane production were observed from soils from the eutrophic region (F1) of the Everglades. In addition, methane production was observed earlier in these microcosms. Microcosms containing soils from the oligotrophic region (U3) of the Everglades produced the lowest amount of acetate and methane. In microcosms with soils from the transition region (F4), however, production rates of methane and acetate were between those observed for the F1 and U3 microcosms. In F1 and F4 microcosms, butyrate accumulated in small amounts and was rapidly consumed. In U3 microcosms, no significant butyrate production was detected. Propionate levels were higher in all control microcosms. F1 and F4 microcosms were more active in terms of propionate production and consumption rates. Higher activities observed in F1 and F4 microcosms may be attributed to higher labile carbon contents of these soils (Table 2-2) that can be utilized immediately by microorganisms when the microcosms established. Related to that, higher TP, TC and MBC may also be indicators of more active bacterial groups and readiness of these groups may be an important factor. To test this possibility and to see the effect of different carbon sources on fermentation and methanogenesis, microcosms containing glucose and cellulose as carbon source were established.

The purpose of the glucose microcosm experiment was to assess the effect of glucose on fermentation product pattern and methane production in Everglades soils.

Glucose concentration was 20 mM, higher than normally expected in natural anaerobic environments. Carbon fractionation in different fermentation products in F1, F4 and U3 soils microcosms spiked with glucose at the end of the experiment are presented in Table 2-7. F1 microcosms (Figure 2-4 A) began to produce methane after approximately 20 days of lag period. In F4 microcosms (Figure 2-4 B), the lag period was 1 week longer than that in F1 microcosms. On the other hand, no methane was produced by U3 microcosms containing glucose (Figure 2-5). This significant difference in methane production indicates that the soils in eutrophic and transition regions in the Everglades possess microbial community or well established microbial associations that can eliminate any excess fatty acids or any other by-products that may be produced as a result of metabolism of high level glucose. Complete butyrate consumption observed in F1 and F4 microcosms supports this conclusion because butyrate consumption refers to syntrophic association involving syntrophs and hydrogen-consuming methanogens. Absence of methane production and absence of acetate and butyrate consumption in U3 microcosms indicates complete inhibition of microorganisms belonging to higher trophic level (syntrophs, hydrogenotrophic and acetoclastic methanogens).

In microcosms in which methane is formed, acetate was produced and consumed. Evidence of active sythrophic associations, meaning fatty acid consumption, in F1 and F4 microcosms strongly indicates that hydrogenotrophic methanogens were also active considering the fact that syntrophs are highly sensitive to hydrogen. Indeed, it was reported that, in F1 soils, the rate of methanogenesis using formate was 2.5 times higher than the rate of acetate consuming methanogenesis (Castro et al. 2004). Total consumption of glucose and similar acetate production trends in the first seven days by

F1 and U3 soils may indicate similar activities of fermentative bacteria in the two soils. MPN data for fermentative bacteria (Table 2-5) supports this conclusion. Significantly higher activities of syntrophic bacteria and methanogenic bacteria were observed in F1 microcosms than U3. A study on sytrophic-methanogenic associations in the Everglades soils indicated that F1 soils contain 100 times higher numbers of hydrogenotrophic methanogens than U3 soils do. Similarly, propionate and butyrate oxidizing sytrophic bacteria were found to be 10 times higher in F1 soils than in U3 soil (Chauhan et al. 2004). These results emphasize the importance of synthrophic and methanogenic activities on fermentation products. This may also explain why U3 glucose microcosm did not show any methanogenic and syntrophic activity under glucose treatment. It is possible that since hydrogenotrophic methanogens are in low numbers, these bacteria could not remove H_2 from the system, thereby inhibiting syntrophy. This, in turn, may result in accumulation of butyrate, and possibly ethanol, hindering activities of other microbial groups. Moreover, feedback mechanisms caused by this situation may direct solventogenic primary fermenters to produce solvents to minimize the toxic effects of high level of fatty acids. These solvents also inhibit microorganisms. Similar responses in pure culture studies were also reported by others (Jones and Woods 1986; Schink 1991; Schink 1997; Conrad 1999).

Cellulose microcosms started producing methane earlier than did glucose microcosms. Carbon fractionation in different fermentation products in F1, F4 and U3 soils microcosms spiked with glucose at the end of the experiment is presented in Table 2-8. Methane production in F1 and F4 cellulose microcosms (Figure 2-6) appeared to be divided into two stages. In the first stage, methane was produced in small amounts

and gradually increased beginning from the first week of the experiment. Later, in the third week for F1 microcosm and the fifth week for F4 microcosm, a sharp increase in methane production was observed. This corresponded with a sharp decline in acetate concentration. It is possible that the first stage is a result of methane production by hydrogenotrophic methanogens and the second stage may be a result of methane producers responding to acetate whose amount is increasing with cellulose decomposition. Similar trends were also occasionally observed in microcosms containing rice roots after a prolonged incubation and it was concluded that the initial stage involved CO_2 mediated methanogenesis (Lehmann-Richter et al. 1999). However, methane formation by hydrogen utilizing methanogens was lower than expected. This is possibly due to activity of homoacetogens competing with hydrogenetrophic methanogenes for hydrogen. Indeed, Drake et al. (1996) reported that homoacetogens are active in Everglades soils.

Interestingly, in the 40^{th} and 50^{th} days in F1 and F4 cellulose microcosms, respectively, a pause in methane production was observed and corresponded with a decline in acetate concentrations. This can be explained by a microbial switch from methanogens operating in higher acetate conditions to those operating in low acetate concentrations. However, the same phenomenon was also observed in cellulose microcosm with benthic periphyton samples in 20^{th} days, where acetate was higher (Chapter 4). Therefore, microbial switch based on acetate concentration alone is not enough to explain this phenomenon. Another possible explanation is sulfate dependent anaerobic methane oxidation by methanogens. This phenomenon has been known for about 30 years based on indirect evidence (Martens and Berner 1977). Recent

developments in molecular and stable isotope techniques also confirmed the existence of this process in nature. Unfortunately, to date, no pure culture or cocultures of microorganisms performing anaerobic methane oxidation have been isolated. There are some theories regarding the mechanisms by which anaerobic methane oxidation proceeds. One theory is that sulfate dependent anaerobic methane oxidation involves sulfate reducers as H_2 sink and methanogens reversing hydrogenotrophic methanogenesis and producing CO_2 and H_2. Another theory suggests that acetoclastic methanogenesis is reversed and acetate and H_2 produced are consumed by sulfate reducing bacteria (Martens and Berner 1977; Valentine 2002; Megonigal et al. 2004). However, these processes depend on the presence of sulfate in the system, sulfate additions in 5 mM concentration to U3 (ridge) and benthic periphyton microcosms in the late stage of the experiment did not provide a conclusive result suggesting whether such archea-bacteria association established in the microcosm. However, it must also be noted that, in theory, not only sulfate but also nitrate, iron (III) and manganese (IV) can be used as alternative electron acceptors during anaerobic methane oxidation. Yet, no supportive evidence has been reported to date (Valentine 2002).

Similar fatty acid production and consumption and methane production patterns were observed in microcosms containing soils from Blue Cypress Marsh. Control microcosms with eutrophic soils (NE) of Blue Cypress Marsh produced higher acetate than oligotrophic soils (BCT) (Figure 2-8). Unlike U3 soils of the Everglades (Figure 2-3), BCT control microcosms did not produce methane, and acetate was not consumed. In NE control microcosms, propionate accumulation was higher than that in BCT

microcosms, and small amounts of butyrate accumulated, and were then consumed in NE microcosms.

In glucose microcosms, acetate accumulated at a similar rate by NE and BCT soils (Figure 2-9). However, NE microcosms consumed acetate earlier and at a faster rate than observed in BCT microcosm. Similarly, methane production was observed approximately 2 weeks earlier in NE microcosms than in BCT microcosms. BCT microcosm did not consume acetate completely, and methane production halted when acetate concentrations stabilized, although acetate never reached zero.

Cellulose microcosms of Blue Cypress Marsh soils showed similar trends in acetate and methane activities compared to the Everglades microcosms (Figure 2-10). NE cellulose microcosms started producing methane approximately 2 weeks earlier than BCT microcosms did, and acetate was consumed earlier by NE soils than by BCT soils, corresponding to the increase in methane. The same phenomenon in which methane production stopped between 40 and 50 days was also observed in Blue Cypress Marsh cellulose microcosms.

In general, propionate was detected in all microcosms at some level. Control and cellulose microcosms with soils from eutrophic regions appeared to produce more propionate than butyrate. A similar trend was reported for rice paddies (Conrad and Klose 1999). It is most likely that the conditions in which available carbon is released slowly to the system promote propionate-producing fermentation pathway. Related to that, propionate may also be produced via an interesting pathway that has not been observed in nature. Conrad and Klose (1999) observed in an experiment in which radioactive compounds were used in microcosms containing rice roots, that bicarbonate and acetate

were incorporated to propionate. They concluded that propionate was produced by reduction of acetate or CO_2. Formation of propionate from acetate is a reversal of syntrophic propionate oxidation and it is known that some sulfate reducing bacteria employs this pathway. Propionate formation from CO_2 and H_2 is also possible. However, propionate production by CO_2 reduction via homoacetogenesis in the environment and an organism performing this pathway has not been reported to date. In cellulose microcosms, only eutrophic soils showed propionate consumption (Figure 2-6 A and 2-10 A), and this occurred after acetate was almost depleted. This raises the question of whether acetate has an impact on propionate consuming bacteria. Indeed, Fukuzaki et al. (1990) demonstrated that acetate has an inhibitory effect on propionate degradation. The importance of acetate on fatty acid degradation was also reported by others (Vanlier et al. 1993; Warikoo et al. 1996; Voolapalli and Stuckey 1999). Voolapalli and Stucky (1999) increased propionate oxidation by increasing acetate-utilizing methanogenes in their study. However, Chauhan et al. (2004) indicated that F1 and U3 soils of the Everglades contain similar number of acetate-utilizing methanogens. Based on this, if acetate were the main controlling factor on propionate degradation in our microcosms, we would have also seen propionate consumption in U3 soils.On the other hand, Chauhan et al. (2004) also reported 100 times higher numbers of hydrogenotrophic methanogens in F1 than in U3 soils. This is an indication that the hydrogen consuming methanogens are among the major players in determination of fermentation production and consumption in Everglades soils.

Effect of Plant Type as Carbon and Nutrient Source on Carbon Cycling

Table 2.9 shows the chemical parameters for plant material used in microcosm experiments. Cellulose and lignin contents of plant material were found to be almost

identical. Hemicellulose content of sawgrass was about 6% higher than that of cattail and ash content were 2 times higher in sawgrass samples than in cattail samples. The biggest difference between plant materials was found in their TN and TP contents. Cattails contain 2 times more TN and 6 times more TP than does sawgrass used in this study.

MPN enumeration (Table 2.10) showed that, at the end of the experiment, there was no significant difference between plant microcosms in terms of cellulolytic bacterial numbers (with no P addition). Interestingly, microcosms with cattail plant material were found to contain 100 times more fermentation bacteria.

When phosphorus was supplied in the liquid media used for plant microcosm, F1 soils show significantly higher methane production with sawgrass than with cattail (Figure 2-11 A). Acetate was also consumed earlier in these microcosms. U3 microcosms (Figure 2-11 B) produced more methane with cattail plant material. However, due to large standard errors it could not be concluded that this difference is significant. Whether TP content of plant materials has an impact on carbon cycling could not be tested since standard liquid media that was used in microcosm experiments already contained this nutrient. Therefore, another microcosm experiment was prepared without any phosphorus addition.

Plant microcosm experiments with no phosphorus gave similar results (Figure 2-12). With F1 soils (Figure 2-12 A), sawgrass microcosms produced more methane than cattail microcosms. Acetate formation and consumption trends in these microcosms were almost identical. Similarly, plant microcosms with U3 soils (Figure 2-12 B) showed almost identical methane production pattern and U3-cattail microcosm accumulated slightly higher acetate concentrations.

A similar trend was also observed in plant microcosms containing eutrophic soil (NE) from Blue Cypress Marsh (Figure 2-13 A). Sawgrass microcosms provided small but significantly higher methane than cattail microcosms. However, oligotrophic soils (BCT) of Blue Cypress March showed significantly higher methane with cattail than with sawgrass (Figure 2-13 B).

In the Everglades, plant material type as carbon and nutrient source appeared to be significant only in F1 soils based on methanogenesis and it seems that sawgrass stimulated carbon cycling. Sawgrass and cattail contain similar amounts of cellulose. However, sawgrass has approximately 6% higher hemicellulose content. Hemicellulose is one of the major components of plants and is utilized by microorganisms as a carbon source (Eriksson et al. 1990). However, cattail was found to contain higher amount of phenolic compounds than does sawgrass (Todd Osborne, personal communication). It is known that phenolic compounds have inhibitory effect on carbon cycling processes (Freeman et al. 2001). It is possible that, in F1 microcosms with cattail, methanogenesis may have been inhibited by phenolic compounds released from cattail material. Higher P content of cattail may not be an important factor in these microcosms due to the fact that F1 soils already have higher P content than U3 soils. However, this does not explain why there is no significant difference in methane production in U3 plant microcosms. U3 soil samples that were used in these experiments were collected from the slough sides of the Everglades where there is less plant biomass. Therefore, microbial populations that can consume various carbon compounds added with plant material may be lower in U3 soils. Higher TP content of cattail also did not produce any significant difference. The

explanation for this can be the possibility that phosphorus is not a limiting factor for methanogenesis in U3 soils.

MPN enumeration of cellulolytic microorganism revealed no significant difference between soil samples (Table 2-10). MPN enumeration for fermentation bacteria, however, indicated significantly higher numbers (100 times) with cattail plant material. This difference may be due to higher Neutral Detergent Soluble Fraction (NDF) content of cattail. NDF represents the soluble fraction of plant material and consist of sugars, pectin, proteins, etc. and these compounds are highly utilizable by fermentation bacteria (Smouter et al. 1995). Therefore, cattail may stimulate fermentative microorganisms more effectively from the beginning of the initial inoculation.

Importance of Carbon in Oligotrophic Everglades Soils

The oligotrophic regions of Everglades are considered to be phosphorus-limited environments, and nutrients, especially phosphorus, are put into these regions by runoff from nearby agricultural areas. With the assumption that differences in microbial communities in Everglades soils are a direct function of phosphorus level in Everglades soils, microcosms containing U3 soils and water were amended with different amounts of phosphorus, and methane formation was monitored. The initial purpose was to monitor microbial changes over time by molecular techniques. However, no significant difference was observed on methane formation and this raised the question whether carbon or another nutrient, but not phosphorus, is the limiting factor for microorganisms in the oligotrophic region. Indeed, cellulose and glucose additions to some of the microcosms resulted in significant increase in methane production while ammonium-N addition had no effect (Figure 2-14). Moreover, plant microcosm experiments (with no phosphorus addition in medium) with U3 soils (Figure 2-12 B), in which 6 times higher amount of

phosphorus was added with cattail plant material, provided the supportive evidence that the phosphorus may not be the first priority for microorganism in the oligotrophic soils of Everglades. However, indirect control of phosphorus on microbial processes is acknowledged considering the fact that type and amount of plant material, hence amount of carbon available to microorganisms, are the functions of phosphorus level in the whole ecosystem (DeBusk and Reddy 1998).

Table 2-1. Moisture and phosphorus parameters for soil samples from the Everglades in Spring, 2002. Provided by the Wetland Biogeochemistry Laboratory.

Sampling Site	Moisture content (%)	Total phosphorus (mg/kg)[b]	Total Inorganic Phosphorus (mg/kg)
F1	92 (1.0)[a]	1110 (352)	366 (128)
F4	93 (1.0)	767 (49)	310 (72)
U3	93 (2.0)	449 (161)	221 (131)
U3 (Ridge)	91 (1.0)	414 (18)	65 (15)

[a] Standard deviations are presented in parentheses.
[b] Concentrations of different substances are expressed per kg (dry weight) of soil.

Table 2-2. Carbon and nitrogen parameters for soil samples from the Everglades in Spring, 2002. Provided by the Wetland Biogeochemistry Laboratory.

Sampling Site	Total Carbon (g/kg)[b]	Microbial Biomass Carbon (mg/kg)	Extractable Total Organic Carbon (mg/kg)	Total Nitrogen (g/kg)	Extractable Ammonium (mg/kg)
F1	446 (24)[a]	7705 (1534)	2404 (204)	28.8 (2.1)	90 (13)
F4	357 (10)	8933 (1529)	2436 (284)	25.3 (2.6)	107 (16)
U3	230 (42)	2627 (128)	1973 (450)	32.8 (2.8)	103 (33)
U3 (Ridge)	462 (9)	2139 (633)	2581 (159)	30.1 (0.4)	68 (9)

[a] Standard deviations are presented in parentheses.
[b] Concentrations of different substances are expressed per kg (dry weight) of soil.

Table 2-3. Moisture and phosphorus parameters for soil samples from the Blue Cypress Marsh Conservation Area in Fall, 2001. Provided by the Wetland Biogeochemistry Laboratory.

Sampling Site	Moisture content (%)	Total phosphorus (mg/kg)[b]	Total Inorganic Phosphorus (mg/kg)
NE	89 (2.0)[a]	897 (110)	114 (44)
BCT	86 (1.0)	519 (77)	67 (23)

[a] Standard deviations are presented in parentheses.
[b] Concentrations of different substances are expressed per kg (dry weight) of soil.

Table 2-4. Carbon and nitrogen parameters for soil samples from the Blue Cypress Marsh conservation area in Fall, 2001. Provided by the Wetland Biogeochemistry Laboratory.

Sampling Site	Total Carbon (g/kg)[b]	Microbial Biomass Carbon (mg/kg)	Extractable Total Organic Carbon (mg/kg)	Total Nitrogen (g/kg)	Extractable Ammonium (mg/kg)
NE	462 (8)[a]	5318 (558)	3128 (250)	24.9 (1.5)	61 (9)
BCT	380 (22)	2115 (340)	2006 (200)	28.3 (2.0)	66 (10)

[a] Standard deviations are presented in parentheses.
[b] Concentrations of different substances are expressed per kg (dry weight) of soil.

Table 2-5. Most probable numbers of cellulolytic and fermentative bacteria in Everglades soils

Soil	Cellulose	Fermentation
F1	$2.39x10^5$ (0.76- 7.60)[a]	$5.42x10^6$ (1.79-14.19)
F4	$3.47x10^5$ (1.17-10.16)	$9.17x10^6$ (2.67-22.01)
U3	$2.43x10^4$ (0.78- 7.40)	$1.72x10^6$ (0.43- 4.97)
U3 (Ridge)	$1.16x10^5$ (0.25- 2.70)	$16.10x10^7$ (3.83-41.03)

[a] Confidence levels (95%) are presented in parentheses.

Table 2-6. Most probable numbers of cellulolytic and fermentative bacteria in Blue Cypress Marsh soils

Soil	Cellulose	Fermentation
Impacted (NE)	$5.42x10^5$ (1.79-14.1)[a]	$5.42x10^6$ (1.79-14.19)
Nonimpacted (BCM)	$2.11x10^4$ (0.65- 6.4)	$2.21x10^6$ (0.56- 7.08)

[a] Confidence levels (95%) are presented in parentheses.

Table 2-7. Carbon fractionation in various fermentation products in Everglades soil microcosms spiked with glucose

	mmole Carbon			
	F1	F4	U3	U3 (ridge)
Acetate	0.06	0.46	2.98	2.19
Butyrate	0.09	0.15	1.18	1.48
Propionate	0.32	0.33	0.37	0.30
Methane	1.42	1.45	0.01	0.01
Total C counted	1.89	2.39	4.54	3.98
Carbon unaccounted	4.11	3.61	1.46	2.02

Table 2-8. Carbon fractionation in various fermentation products in Everglades soil microcosms spiked with cellulose

	mmole Carbon			
	F1	F4	U3	U3 (ridge)
Acetate	0	0.01	0.32	0.31
Butyrate	0	0	0	0.04
Propionate	0.01	0.64	0.43	0.88
Methane	1.38	1.23	0.48	0.72
Total C counted	1.39	1.88	1.23	1.95
Carbon unaccounted	4.85	4.36	5.01	4.29

Table 2-9. Biochemical parameters for plant material used in microcosm experiments. Provided by the Wetland Biogeochemistry Laboratory.

Plant Material	Cellulose (%DW)	Hemicellulose (%DW)	Lignin (%)	Ash (%DW)	NDF[a] (%DW)	TN (%DW)	TP (%DW)
Cattail	40.3	13.8	15.3	1.5	29.3	0.75	0.035
Sawgrass	41.8	19.3	15.2	3.7	20.1	0.40	0.006

[a] Neutral detergent soluble fraction

Table 2-10. Most probable numbers of cellulolytic and fermentative bacteria in plant microcosms containing soils from F1 and U3 regions of the Everglades

Microcosm	Cellulose	Fermentation
F1-Cattail	2.40×10^{8} (0.48- 9.65)[a]	2.14×10^{11} (0.34 - 9.00)
F1-Sawgrass	4.27×10^{7} (1.03-13.80)	3.05×10^{9} (0.50 -11.00)
U3-Cattail	9.33×10^{7} (2.06-27.10)	4.62×10^{11} (1.16-15.00)
U3-Sawgrass	9.33×10^{7} (2.06-27.10)	3.05×10^{9} (0.50 -11.00)

[a] Confidence levels (95%) are presented in parentheses.

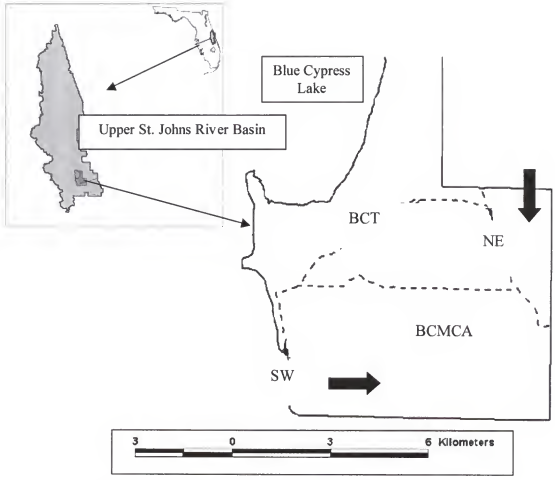

Figure 2-1. Map of Blue Cypress Marsh Conservation Area (BCMCA)

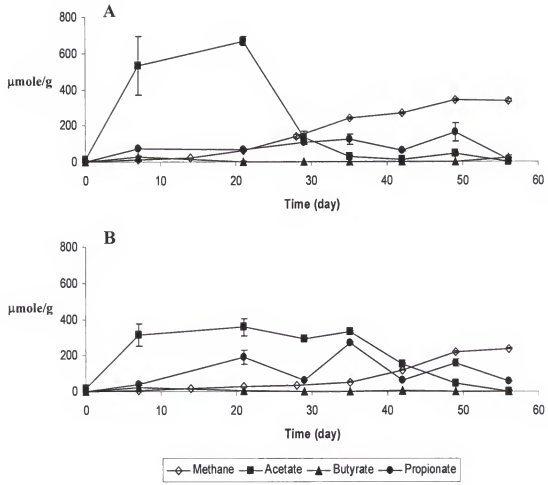

Figure 2-2. Acetate, butyrate, propionate and methane production potentials of eutrophic and transition soils of the Everglades in microcosms containing no external carbon source. Error bars represent standard errors based on 5 replicates. A) F1-control microcosm. B) F4-control microcosm.

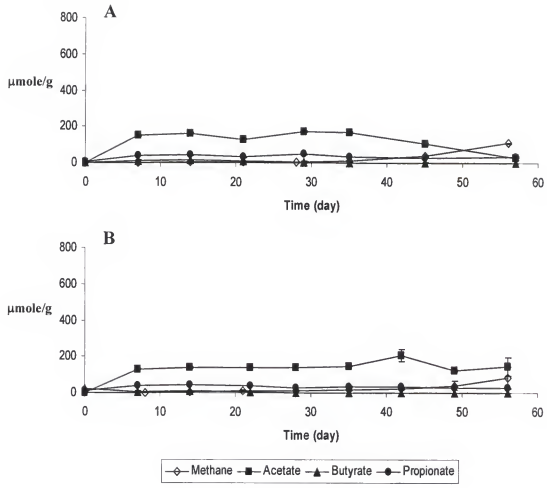

Figure 2-3. Acetate, butyrate, propionate and methane production potentials of oligotrophic soils of the Everglades in microcosms containing no external carbon source. Error bars represent standard errors based on 5 replicates. A) U3-control microcosm. B) U3 (Ridge)-control microcosm.

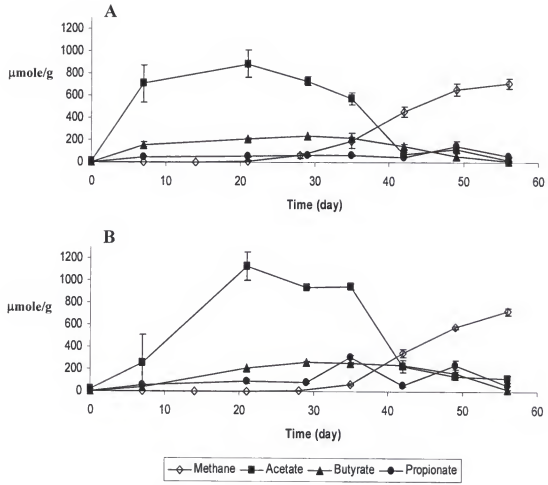

Figure 2-4. Effect of glucose on fermentation products and methane production in eutrophic and transition soils of the Everglades. Error bars represent standard errors based on 5 replicates. A) F1-glucose microcosms. B) F4-glucose microcosms.

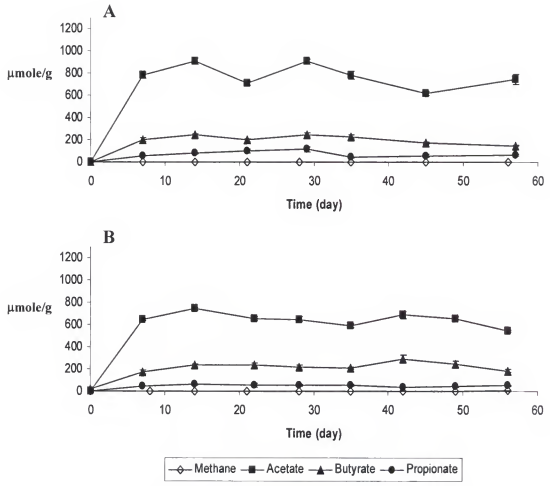

Figure 2-5. Effect of glucose on fermentation products and methane production in oligotrophic soils of the Everglades. Error bars represent standard errors based on 5 replicates. A) U3-glucose microcosms. B) U3 (Ridge)-glucose microcosms.

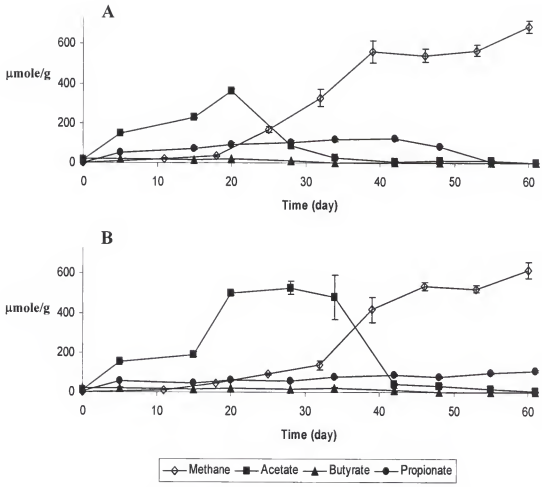

Figure 2-6. Effect of cellulose on fermentation products and methane production in eutrophic and transition soils of the Everglades. Error bars represent standard errors based on 3 replicates. A) F1-cellulose microcosms. B) F4-cellulose microcosms.

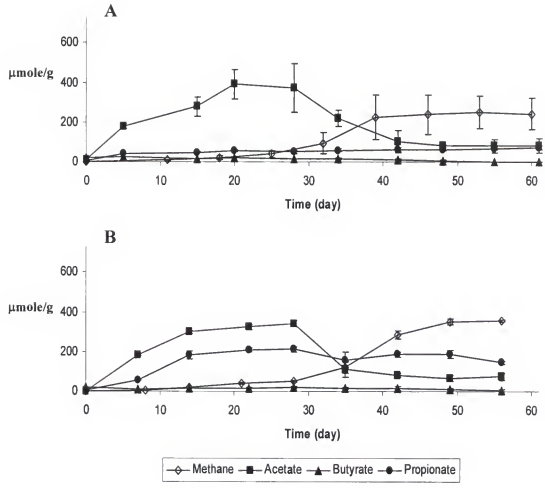

Figure 2-7. Effect of cellulose on fermentation products and methane production in oligotrophic soils of the Everglades. Error bars represent standard errors based on 3 replicates. A) U3-cellulose microcosms. B) U3 (Ridge)-cellulose microcosms.

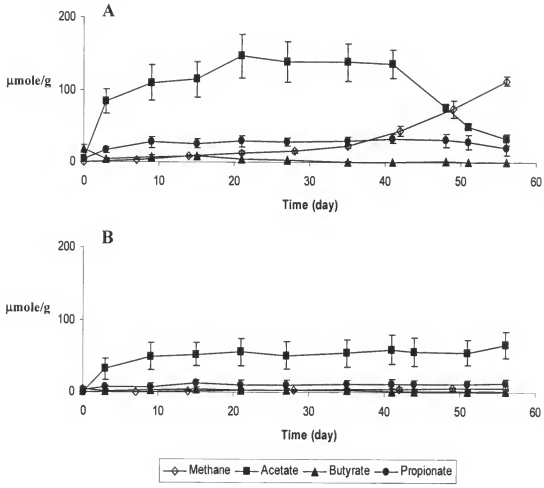

Figure 2-8. Acetate, butyrate, propionate and methane production potentials of high-nutrient and low-nutrient soils of the Blue Cypress Marsh in microcosms containing no external carbon source. Error bars represent standard errors based on 3 replicates. A) NE-control microcosm. B) BCT-control microcosm.

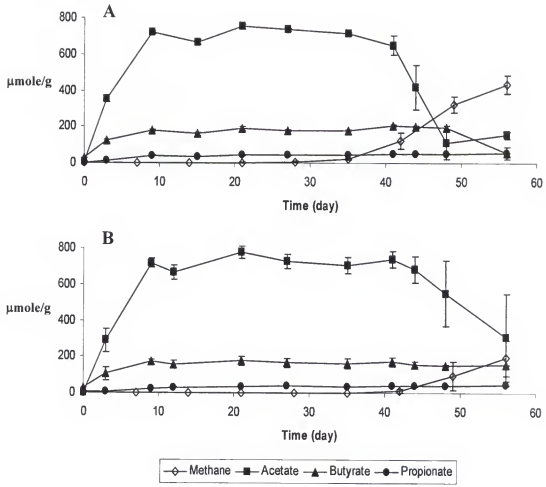

Figure 2-9. Effect of glucose on fermentation products and methane production in high-nutrient and low-nutrient soils of the Blue Cypress Marsh. Error bars represent standard errors based on 3 replicates. A) NE-glucose microcosms. B) BCT-glucose microcosms.

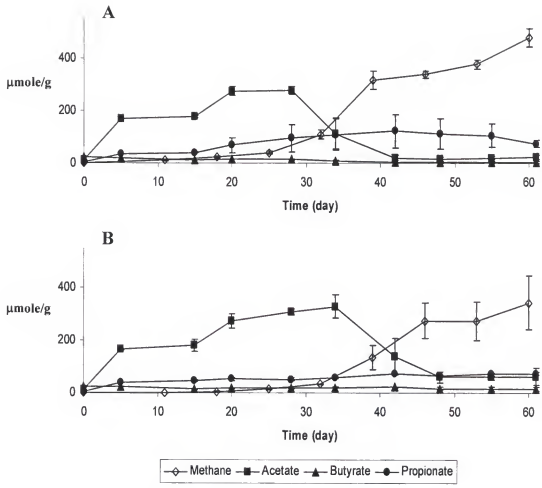

Figure 2-10. Effect of cellulose on fermentation products and methane production in high-nutrient and low-nutrient soils of the Blue Cypress Marsh. Error bars represent standard errors based on 3 replicates. A) NE-cellulose microcosms. B) BCT-cellulose microcosms.

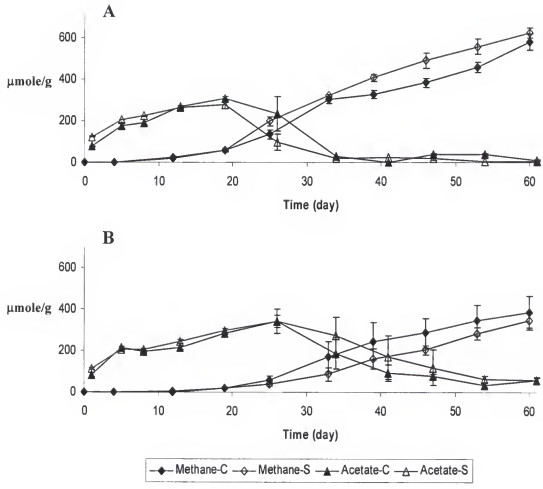

Figure 2-11. Effect of plant materials on fermentation products and methane production in Everglades soils. Error bars represent standard errors based on 3 replicates. A) F1-plant microcosm. B) U3-plant microcosm. (C, cattail; S, sawgrass)

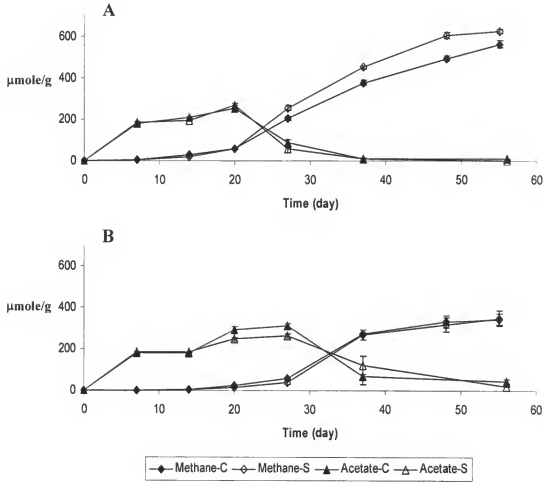

Figure 2-12. Effect of plant materials on fermentation products and methane production in Everglades soils in the absence of phosphorus in the media. Error bars represent standard errors based on 5 replicates. A) F1-plant microcosm. B) U3-plant microcosm. (C, cattail; S, sawgrass)

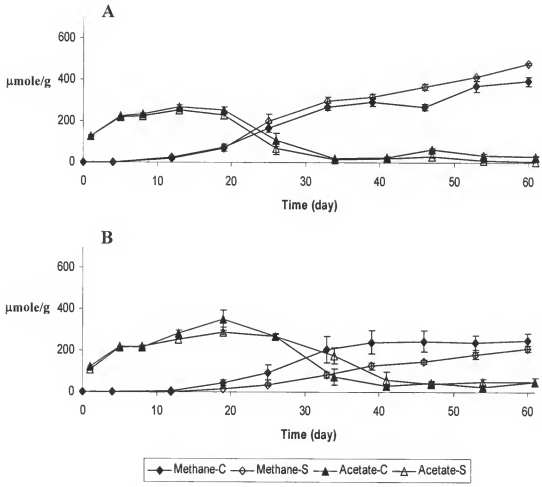

Figure 2-13. Effect of plant materials on fermentation products and methane production in Blue Cypress Marsh soils. Error bars represent standard errors based on 3 replicates. A) NE-plant microcosm. B) BCT-plant microcosm. (C, cattail; S, sawgrass)

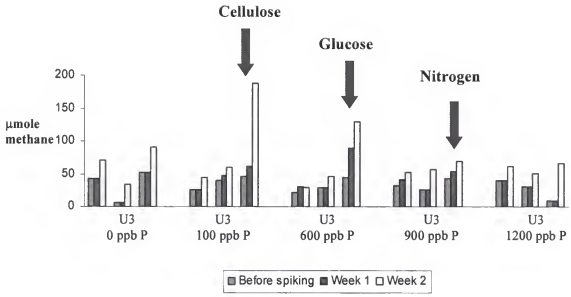

Figure 2-14. Effect of cellulose, glucose and nitrogen (NH_4-N) additions to microcosm with U3 soils under different level of phosphorus amendments.

CHAPTER 3
COMPOSITION OF CELLULOLYTIC AND FERMENTATIVE *Clostridium* COMMUNITIES IN THE EVERGLADES SOILS

Due to the fact that conventional techniques have limited ability to reveal the true importance of microorganisms in environment, and that most of the information available on bacteria comes from studies employing these techniques, scientists still do not have a complete understanding of many bacterial functions in a particular environment, or of the significance of changes in bacterial communities. One of the bacterial groups that requires attention in anaerobic environments is the genus *Clostridium*. Species belonging to the *Clostridium* group have been isolated and used since the early 1900s in energy and chemical industries due to their ability to convert sugars to solvents in high quantity. With the energy crises in the 1970s, the importance of cellulose degradation as a means for food, fuel, and chemical supply was realized and cellulolytic *Clostridium* has become one of the most studied bacterial groups. However, almost all of these studies were conducted with pure cultures under controlled conditions. Even though they provided highly valuable information, these studies did not provide clues about the ecology of *Clostridium*. PCR, sequencing and phylogenetic analysis that have become commonly used techniques provide most needed information to microbial ecologists.

With nutrient impact coming from nearby agricultural areas, the Everglades provides opportunity to investigate effects of nutrient loading on microbial communities. The impacts of nutrient loading on some microbial processes, such as methanogenesis and respiration, have been well studied. However, few reports are available on the impact

of nutrient loading on ecology of microbial groups in Everglades soils (Castro et al. 2002, 2004; Chauhan et al. 2004). No report on *Clostridium* assemblages, one of the major cellulolytic and fermentative bacterial groups in anaerobic environments, in Everglades soils has been published to date.

We are interested in the *Clostridium* group because previous studies on various anaerobic environments emphasized the importance of this group, especially species belonging to clusters I, III, IV and XIV, which are involved in cellulose degradation and fermentation. Moreover, our initial experiments with Everglades soil samples yielded sequences, all of which are closely related to *Clostridium*.

The objective of this chapter is to investigate and compare the composition of cellulolytic and fermentative organisms, namely *Clostridium* species, with molecular techniques targeting 16S rRNA gene. In addition, application of T-RFLP (Terminal Restriction Fragment Length Polymorphism) to study the distribution of *Clostridium* species in Everglades soils is explored.

Materials and Methods

Site Characteristics, Sampling and Biogeochemical Characterization

Soil samples were collected from eutrophic, transition and oligotrophic regions of the Everglades in Spring, 2002. Soil sample collection and preparation methods were previously described in Chapter 2. After replicate soils samples were manually mixed to create a single composite sample representing 0 to 10 cm soil layer for each region, subsamples intended for DNA analysis were stored in -70^{o}C until analysis.

Soil DNA Extraction

DNA was extracted from soil samples, MPN tubes and enrichment vials by using Ultra Clean Soil DNA kit (MoBio, Solana Beach, CA) according to manufacturer's

instructions. In the initial phase of the DNA extraction process from MPN tubes and enrichment vials, pellets were obtained from 1 to 2 mL cultures and suspended in the kit's bead solution. After extraction, DNA was analyzed on 0.7 to 1% agarose gel electrophoreses in Tris-Acetate-EDTA (TAE) buffer. DNA was stored at –20°C until further analysis.

Polymerize Chain Reaction (PCR) Amplification

Various primer sets were used. Primer names, sequences, annealing temperatures, and target groups for amplification by the polymerase chain reaction (PCR) are presented in Table 3-1. For DNA from MPN and enrichment cultures, universal bacterial primers targeting the 16S rRNA gene were used. For soil DNA, specific primers targeting 16S rRNA genes of *Clostridium* species belonging to clusters I, III, IV and XIV were used. PCR reaction mixtures contained 10 µL of HotStarTaq master mix (Qiagen, Valencia, CA), 7 µL of distilled H_2O, 1 µL of each primers (10 pmol/µL) and 1 µL of diluted DNA solution. For universal primers, PCR cycling was performed at 94°C for 30 seconds for denaturation and at 72°C for 30 seconds for chain extension. For *Clostridium*-specific primers, similar temperature conditions were performed for 1 minute. Annealing was performed for 30 seconds, and 1 minute, for universal bacterial and *Clostridium*-specific primers, respectively, at temperatures shown in Table 3-1. Reaction mixtures were subjected to 35 cycles for universal bacterial primers and 40 cycles for *Clostridium*-specific primers in a Perkin-Elmer Model 2400 Thermal Cycler (Perkin-Elmer, Norwalk, CT). An initial activation step of 95°C for 15 minutes was required for HotStarTaq master mix. An additional seven minutes were added for chain extension at the end of reactions.

Cloning of 16S rRNA Genes and RFLP Analysis

Fresh PCR products were ligated into pCRII-TOPO cloning vector (Invitrogen, Carlsbad, CA) and transformed into chemically competent *E. coli* cells (TOP10F') according to the vendor's instructions. Individual colonies were screened by direct PCR amplification and restriction fragment length polymorphism (RFLP) analysis was performed using digestion enzymes *Hha*I+*Eco*RV for Cluster I, *Alu*I for Cluster III, and *Msp*I for Cluster IV and XIVab clones. Selection of digestion enzymes for RFLP was based on *in silico* analysis of previously identified 16S rRNA genes of *Clostridium* species in National Center Biotechnology Information (NCBI) database using CloneMap software (version 2.11, CGC Scientific Inc, Ballwin, MO). Digestion reactions were analyzed in 2% agarose gels.

Sequencing and Phylogenetic Analysis

Selected clones that were representatives of different digestion patterns were sequenced by University of Florida's Interdisciplinary Center for Biotechnology Research core sequencing facility. Sequences were compared with previously identified sequences in NCBI database using BLAST (Altschul et al. 1990). The sequences obtained in this study were initially aligned with closely matched sequences from NCBI database using the Pileup function of GCG Package (Accelrys Inc., San Diego, CA) and adjusted manually with ClustalX version 1.8 (Thompson et al. 1997). Phylogenetic trees were generated with TREECON (Van de Peer and De Wachter 1994; Van de Peer and De Wachter 1997) using a neighbor-joining method. Bootstrap analysis was performed with 100 resamplings of the DNA sequences to estimate the confidence of tree topology.

Terminal Restriction Fragment Length Polymorphism (T-RFLP) Analysis

T-RFLP analysis was conducted on soil samples collected from eutrophic, transition, and oligotrophic regions of the Everglades between April 2001 and August 2002. DNA was extracted from soil samples by using Ultra Clean Soil DNA kit (MoBio, Solana Beach, CA) according to manufacturer's instruction. For PCR, primers Erec-0482-a-S-19 and Ccoc-1112-a-A-19 (Table 3-1) targeting 16S rRNA gene of *Clostridium* Cluster XIV species were used. The forward primer Erec-0482-a-S-19 was labeled with 6-FAM (6-carboxyfluorescein) by the vendor (Invitrogen, Carlsbad, CA). The same PCR cycling conditions, except annealing temperature, were used as described in previous subtitles. The annealing temperature was set to be 53°C instead 55°C. PCR reaction mixture contained 25 μL of HotStarTaq master mix (Qiagen, Valencia, CA), 17.5 μL of distilled H_2O, 2.5 μL of each primer (10 pmol/mL) and 2.5 μL of diluted DNA solution. After confirming the expected PCR product size by electrophoresis in 0.7% agarose gel, products were cleaned and concentrated by using QIAquick PCR purification kit (Qiagen, Valencia, CA) to 30 μL. Between 100 and 150 ng of amplification product were digested with selected restriction enzyme according to vendor's instruction (Promega, Madison, WI). Selection of digestion enzyme for T-RFLP was based on *in silico* analysis of *Clostridium* clone sequences obtained from soil samples by using CloneMap software (version 2.11, CGC Scientific Inc, Ballwin, MO). From digestion reactions, between 1 and 1.5 μl aliquots were processed by University of Florida's Interdisciplinary Center for Biotechnology Research core sequencing facility. T-RFLP analysis was conducted manually by scoring presence and absence of the peak corresponding to expected fragment size. Statistical data analysis was done using SAS software (SAS Institute Inc.,

Cary, NC). Duncan's Multiple Range Test was used to determine significance of differences in T-RFLP data between soil samples.

Results and Discussion

Phylogenetic Analysis of Cloned *Clostridium* 16S rRNA Gene Sequences

PCR amplification using primers targeting *Clostridium* Cluster I species produced the expected 820 bp fragment. DNA from randomly selected clones was amplified with Cluster I specific primers and amplicons were digested with restriction enzymes *Hha* I + *Eco*R V. A total of 13 different RFLP patterns were observed in soils and F1 and F4 soils exhibited greater diversity than U3 soil. Interestingly, soil samples from the transition region (F4) showed the greatest number of phylotypes. Rarefaction curves for Cluster I clones approached the plateau of a complete clone library indicating that there is no need for additional clone selection and screening (Figure 3-1). Representative clones from each RFLP pattern were sequenced and a phylogenetic analysis was conducted for *Clostridium* Cluster I. The phylogenetic tree (Figure 3-2) revealed a separate branch from known Cluster I species. This novel branch only included sequences from eutrophic and oligotrophic samples, all sequences from transition site clustered with the known Cluster I species.

PCR primers targeting *Clostridium* Cluster III, the cellulolytic cluster, produced a 720 bp fragment. Clones carrying this fragment were randomly chosen and amplicons were digested with the restriction enzyme *Alu*I, giving total of six distinct digestion patterns. U3 soil appeared to have greater diversity in Cluster III community than F1 and F4 soils (Figure 3-3). However, the phylogenetic tree of Cluster III containing representative clone sequences did not show any specific clustering based on origin of soil samples (Figure 3-4).

RFLP analysis of the 580 bp fragment amplified by *Clostridium* Cluster IV specific 16S rRNA gene primers indicated the presence of a total of 4 patterns in these soils. F1 soils appeared to have a higher degree of diversity than F4 and U3 soils (Figure 3-5). Branching in the phylogenetic tree of Cluster IV, in general, did not correspond with RFLP patterns. However, only U3 sequences were placed in the branch containing cellulolytic Cluster IV species such as *C. cellulose*, *Ruminococcus albus* and *R. flavefaciens* (Figure 3-6).

Clostridium Cluster XIV primers produced a 620 bp fragment from DNA isolated from soil samples. RFLP patterns produced by restriction enzyme *Msp* I indicated limited diversity of *Clostridium* Cluster XIV in oligotrophic soil samples. Only 2 patterns were associated with U3 soil (Figure 3-7). These patterns corresponded to sequences belonging to the XIVa branch in Cluster XIV phylogenetic tree (Figure 3-8). F1 and F4 soils show similar diversity in terms of number of RFLP patterns observed, and rarefaction indicated that all RFLP groups were covered by the analysis (Figure 3-7). Therefore, no additional clones were screened.

Due to the fact that genus *Clostridium* is a phylogenetically diverse group, there is no single PCR primer set targeting all *Clostridium* species. Therefore, 16S rRNA gene primers based on *Clostridium* clustering system established by Collins et al. (1994) are the only non-culture-based means to investigate species within each cluster. However, this brings problems such as distinguishing different phylotypes by RFLP technique in a particular cluster since sequences in that cluster are similar to each other in some degree. Considering the fact that the technique is based on presence and absence, and location of restriction enzyme cutting sites on PCR amplicons, RFLP analysis based on a single

enzyme may place highly similar species in different groups since sequences on enzyme cutting sites can change between strains without effecting rest of the sequences or vise-versa. Therefore, RFLP must be considered as a comparative method in assessment of diversity but not as a main phylogenetic approach (Barcenilla et al. 2000). Among *Clostridium* clusters that were investigated here, only RFLP pattern analysis of Cluster XIV corresponded with branching in cluster XIV phylogenetic tree showed clear separation between cluster XIVa and XIVb. This differentiation is not surprising since it was reported that Cluster XIV may be separated into two individual clusters in the future (Collins et al. 1994; Stackebrandt and Hippe 2001). However, for other clusters studied, RFLP patterns and phylogenetic analysis did not corresponded with each other.

Cluster I constitutes one of the largest *Clostridium* clusters based on 16S rRNA gene sequences. It contains the type species, *Clostridium butyricum*, and is considered as the core cluster for the genus *Clostridium*. Cluster I contains species that are metabolically versatile, e.g. cellulolytic, saccharolytic and proteolytic members and specialists. However, in general, the mostly saccharolytic aspect of this cluster has been emphasized in ecological studies (Weber et al. 2001). The phylogenetic tree of Cluster I clones obtained from Everglades soils indicates that eutrophication impacted Cluster I assemblage structure (Figure 3-2). The presence of F1 and U3 sequences and absence of F4 sequences in the novel branch implies that Cluster I species composition in transition region, but not oligotrophic region, significantly differs from that in the eutrophic region. In terms of biogeochemical parameters, however, F4 region is more similar to F1 region than is U3 region (Tables 2-1 and 2-2). It is possible to speculate that in the transition site, the system (processes in carbon cycling including syntrophy and methanogenesis)

may be out of balance, adjusting to the new nutrient rich status. During this adjustment period, some of the bacteria may become dormant waiting for the balanced system due to unfavorable conditions for those species. F1 and U3 regions represent the nutrient-rich and nutrient-poor ends of the balanced system, thereby, presence of these sequences in the novel branch. Due to the fact that there is no previously identified Cluster I species located in the novel branch, it is difficult to reach a definitive conclusion about why this difference occurs.

Clones located in the branch with known Cluster I species were distributed almost evenly. Sequence similarity of clones to known species in this branch ranges between 95% and 99%. The sequences showing 98% or higher similarity may function in a similar way. Clones grouped with species such as *C. quinii*, *C. butyricum*, *C. acetobutyricum*, and *C. saccharobutyricum* are likely to take an important role in fermentation of various carbohydrates released from cellulose or other polymeric carbon sources in these soils. Clones T26 and T24 sequences were 99% similar to *C. magnum*, which can perform both homoacetogenesis and carbohydrate fermentation (Karnholz et al. 2002). Clones U111, U105, and U107 grouped with *C. tunisiense* and *C. argentinense*, which can ferment proteins but not sugars (Suen et al. 1988; Thabet et al. 2004). *Clostridium tunisiense* also has the ability to use elemental sulfur as terminal electron acceptor (Thabet et al. 2004). Clone T2 sequences showed 98% similarity with *C. bowmani* that was isolated from Antarctic fresh water lake. This species was found to be psychrophilic and saccharolytic (Spring et al. 2003). The presence of various Cluster I species was also observed during the rice straw degradation in rice paddy soils and 24% of all eubacterial cells belonged to *Clostridium* Cluster I species (Weber et al. 2001). Based on their findings, these scientists

assumed that Cluster I species are responsible for fermentation of rice straw hydrolysis products in rice paddy environments. However, no Cluster I sequence was recovered from landfill and rumen fluid environments (Van Dyke and McCarthy 2002), and only low numbers of Cluster I species were detected in human fecal samples (Franks et al. 1998), suggesting that the presence of Cluster I species is a function of environmental conditions.

Potential impact of eutrophication was also observed in *Clostridium* Cluster XIV clones. Sequences from plant microcosms were also included in the phylogenetic tree to investigate whether type of plant material influences *Clostridium* composition.

The phylogenetic tree of Cluster XIV revealed absence of Cluster XIVb species in oligotrophic samples (Figure 3-8). Sequences from plant microcosms also show the similar distribution in the phylogenetic tree, supporting the data from soil samples. Distribution of sequences from plant microcosms also indicates that type of plant material is not the main factor controlling Cluster XIV community structure. All sequences, with the exception of clone FS2, were 96% or less similar to known Cluster XIV sequences, indicating that the Everglades soils accommodate novel *Clostridium* species. Cluster XIV is the second largest *Clostridium* cluster. Unlike Cluster I, Cluster XIV also contains species belonging to other genera such as *Ruminococcus*, *Eubacteria*, *Syntrophococcus*, *Roseburia*, and *Epulopiscium*. Cluster XIVa species are versatile in their ability to utilize various carbohydrates, including polymeric carbon sources such as cellulose and xylan. Xylans, like cellulose, constitute a major part of plant material and, unlike cellulose, form complex polymers called hemicelluloses (Uffen 1997). In Cluster XIVa, *C. populeti* can degrade xylan, cellulose, and glucose, and produce butyrate and lactate as major products

(Sleat and Mah 1985). Its close relative, *C. xylanovorans*, can utilize xylan but not cellulose (Mechichi et al. 1999; Warnick et al. 2002). On the other hand, *Eubacterium xylanophilum* can ferment xylan and cellobios but cannot grow on glucose or cellulose (Vangylswyk and Vandertoorn 1985), in contrast to *Eubacterium cellulosolvens* that can utilize cellulose but not xylan (Vangylswyk and Vandertoorn 1986). *Clostridium aerotolerans* (Vangylswyk and Vandertoorn 1987; Chamkha et al. 2001) isolated from sheep rumina and *Roseburia intestinalis* (Duncan et al. 2002) isolated from human faeces were found to be xylan degraders, whereas *C. celerecrescens* can utilize both cellulose and xylan (Palop et al. 1989; Palop et al. 1991; Chamkha et al. 2001).

Cluster XIVb contains species from various environments. *Epulopiscium fishelsoni* is a giant bacterium found in intestinal tract of surgeonfish and has not been cultured in laboratory, therefore, its metabolic characteristics are still under investigation (Angert et al. 1993; Angert and Clements 2004). *Clostridium lentocellum*, isolated from a river sediment containing paper-mill waste, has ability to utilize cellulose, xylan and glucose (Murray et al. 1986). In a separate branch, *C. colinum*, the causative agent of ulcerative enteritis in birds, grouped with *C. piliformi* causing Tyzzer's Disease in animals. *C. colinum* was determined to be a glucose fermenting species (Berkhoff 1985). *Clostridum lactatifermentans*, isolated from the caecum of a chicken, grouped with *C. propionicum*, first discovered in a salt-water environment, and *C.neopropionicum*, first recovered from an anaerobic waste digester. These species utilize lactate. Glucose, however, is utilized by *C. lactatifermentans* but not *C. propionicum*. *C. neopropionicum*

may or may not utilize glucose since there are conflicting reports (Cardon and Barker 1946; Janssen 1991; Tholozan et al. 1992; van der Wielen et al. 2002).

Phylogenetic analysis of Cluster XIV sequences (Figure 3-8) obtained from Everglades soils and information gained from members of this cluster strongly indicates that Cluster XIV species are present and possibly active in degradation of polysaccharides in Everglades soils. Similar conclusions were reached for rice paddy soils (Hengstmann et al. 1999; Weber et al. 2001). Cluster XIV species were also detected in high quantity in strict vegetarian human fecal samples, leading to speculation that these species may have an important role in fiber degradation in intestinal tract of vegetarians humans (Hayashi et al. 2002). Van Dyke and McCarty (2002) also detected presence of Cluster XVIa in a test cell reactor containing municipal solid waste. In a methanogenic landfill leachate bioreactor, however, Cluster XIVa clones were associated only with glucose fermentation released from cellulolytic processes (Burrell et al. 2004). Studies reporting similar observations are available (Franks et al. 1998; Wang et al. 2003).

The phylogenetic tree of *Clostridium* Cluster III did not show any specific branching based on origin of soil samples (Figure 3-4). Based on the fact that Cluster III is the only *Clostridium* cluster that solely consists of cellulolytic species, it is possible to speculate that eutrophication in Everglades soils does not have a significant effect on composition of the cellulolytic *Clostridium* assemblage. However, *Clostridium* Cluster IV phylogenetic tree shows an interesting sequence distribution that may not support this statement (Figure 3-6). Oligotrophic soil clones U2, U3, and U24 grouped with *C. cellulosi*, a thermophilic cellulolytic species. This group is located in a branch

containing other cellulolytic species such as *Ruminococcus flavefaciens* and *R. albus* (Rainey and Janssen 1995). On the other hand, clones F11, T1, T18, and U1 grouped with noncellulolytic species such as *R. bromii*, *C.leptum*, *C. sporosphaeroides* and *C. methylpentosum*. More interestingly, other clones from F1 and F4 soils clustered with *C. orbiscindens*, an asaccharolytic species (Winter et al. 1991; Schoefer et al. 2003). It is tempting to speculate that cellulolytic species in Cluster IV prefer oligotrophic soils whereas soils in eutrophic and transition regions favor noncellulolytic Cluster IV species. However, presence of these species in U3 soil may not necessarily be attributed to their cellulolytic capability. *Clostridium cellulosi*, the closest relative to these clones, ferments more carbohydrates than do other *Clostridium* species in the Cluster IV phylogenetic tree (Yanling et al. 1991). Clones U2, U3, and U24 may exhibit similar features which may provide great advantage to these species in nutrient-limited environments such as U3 soils.

Analysis of T-RFLP for *Clostridium* Cluster XIV Species

Based on results from phylogenetic analysis and due to the observation of distinct branching in phylogenetic trees according to origin of sequences, Cluster I and Cluster XIV clone sequences obtained from soil samples were subjected to *in silico* digestion with more than 40 different restriction enzymes. None of the enzymes tested distinguished Cluster I sequences based on their places in the phylogenetic tree. However, enzyme *Hinc*II provided clear separation between Clusters XIVa and XIVb. When digested with *Hinc*II, Cluster XIVb sequences produced a fragment of approximately 379 bp, whereas cluster XIVa sequences did not produce a similarly sized fragment. In the Cluster XIV phylogenetic tree, none of oligotrophic sequences was placed in Cluster XIVb branch. Using these findings, T-RFLP method was conducted

with Cluster XIV PCR amplicons from DNA extracted from eutrophic, transition and oligotrophic soils collected between April 2001 and August 2002. The results of T-RFLP analysis, including the Duncan test, are presented in Table 3-2. A significant difference ($p<0.05$) was observed between sites based on presence and absence of the peak corresponding to the 379 bp fragment. Thirty-two, 23 and 18 soil samples from eutrophic, transition, and oligotrophic regions, respectively, that yielded positive PCR amplification were included in the statistical analysis. All soil samples from eutrophic and transition regions showed the presence of the expected peak whereas only 39% of the soil samples from the oligotrophic region were counted as positive. In other words, the T-RFLP method distinguished soils samples based on their origins.

It is necessary to acknowledge the effect of primer bias and length of PCR fragment on T-RFLP results. Shorter PCR fragments may not contain enough restriction sites, leading to failure to resolve different species present in samples (Wu et al. 2004). This is especially important for *Clostridium*, since Cluster XIV consists of closely related *Clostridium* species and PCR fragment used for T-RFLP in this study is only approximately 620 bp. New primers that may be designed in the future for this cluster may target larger fragments and provide more detailed T-RFLP data for Everglades soils.

There are several reports, mainly on human digestive tracts, citing detection and monitoring of *Clostridium* species (especially Cluster XIVa) by T-RFLP method (Hayashi et al. 2002; Wang et al. 2004). However, these studies focus on general microbial habitat in those environments and employ general bacterial primers rather than focusing specifically on *Clostridium* using Clostridial primers. To our knowledge, no

report has been published regarding utilization of T-RFLP specifically for *Clostridium* species.

Table 3-1. Primers and annealing temperatures used in this study

Primer (sequence 5'-3')[a]	Target Gene [b]	Annealing Temp.(°C)	Reference
27F (AGAGTTTGATCMTGGCTCAG)	Universal 16S rDNA gene	58	(Lane 1991)
1492R (TACGGYTACCTTGTTACGACTT)	Universal 16S rDNA gene		(Lane 1991)
Chis-0150-a-S-23 (AAAGGRAGATTAATACCGCATAA)	*Clostridium* Cluster I 16S rDNA gene	58[c]	(Franks et al. 1998)
Cbot-0983-a-A-21 (CARGRGATGTCAAGYCYAGGT)	*Clostridium* Cluster I 16S rDNA gene		(Van Dyke and McCarthy 2002)
Cther-0650-a-S-23 (TCTTGAGTGYYGGAGAGGAAAGC)	*Clostridium* Cluster III 16S rDNA gene	60	(Van Dyke and McCarthy 2002)
Cther-1352-a-A-19 (GRCAGTATDCTGACCTRCC)	*Clostridium* Cluster III 16S rDNA gene		(Van Dyke and McCarthy 2002)
Clos-0561-a-S-17 (TTACTGGGTGTAAAGGG)	*Clostridium* Cluster IV 16S rDNA gene	60	(Van Dyke and McCarthy 2002)
Clept-1129-a-A-17 (TAGAGTGCTCTTGCGTA)	*Clostridium* Cluster IV 16S rDNA gene		(Van Dyke and McCarthy 2002)
Erec-0482-a-S-19 (CGGTACYTGACTAAGAAGC)	*Clostridium* Cluster XIVab 16S rDNA gene	55	(Franks et al. 1998)
Ccoc-1112-a-A-19 (TGGCTACTRDRVAYARGGG)	*Clostridium* Cluster XIVab 16S rDNA gene		(Van Dyke and McCarthy 2002)

[a] Y, T/C; V, G/C/A; R, A/G.
[b] Based on clustering system described by Collins et al. (1994).
[c] Originally reported as 65°C.

Table 3-2. Result of T-RFLP application for soil samples from the Everglades

	F1	F4	U3
Number of samples	32	23	18
Positive T-RFs (%)	100	100	39
Negative T-RFs (%)	-	-	61
Duncan Classification*	A	A	B

* Soils in the same Duncan classification group are not significantly different ($p<0.05$)

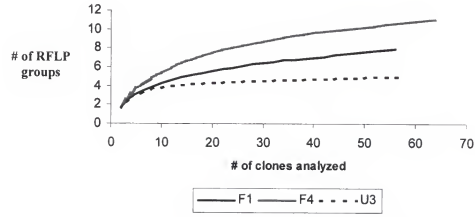

Figure 3-1. Rarefaction analysis for *Clostridium* Cluster I clone libraries for soil samples from the Everglades.

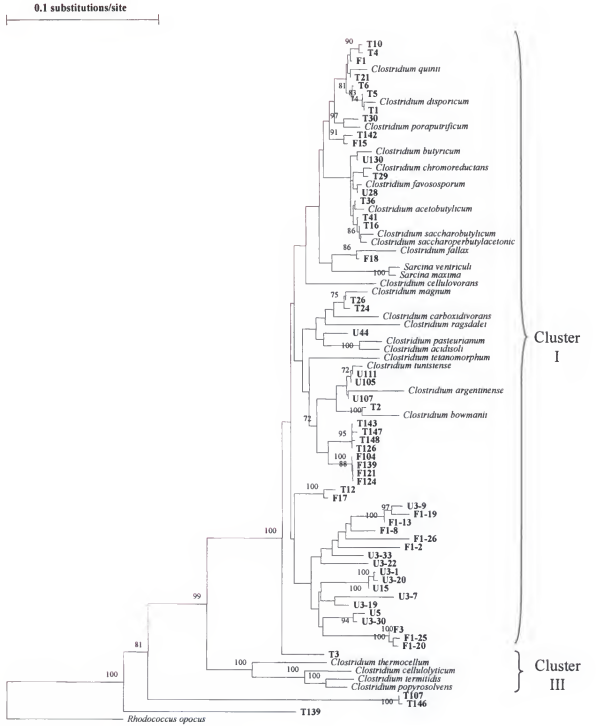

Figure 3-2. Phylogenetic tree of *Clostridium* Cluster I 16S rRNA gene clone sequences obtained from Everglades soils (F1, F; F4, T; U3, U). Numbers at branch points refer to bootstrap analysis based on 100 resampling.

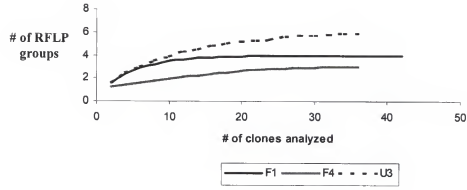

Figure 3-3. Rarefaction analysis for *Clostridium* Cluster III clone libraries for soil samples from the Everglades.

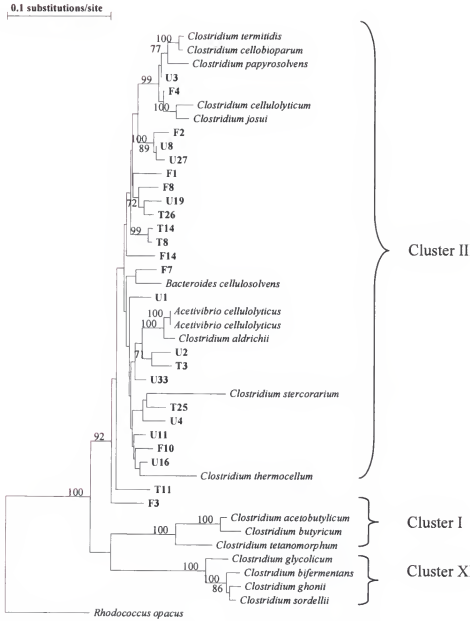

Figure 3-4. Phylogenetic tree of *Clostridium* Cluster III 16S rRNA gene clone sequences obtained from Everglades soils (F1, F; F4, T; U3, U). Numbers at branch points refer to bootstrap analysis based on 100 resampling.

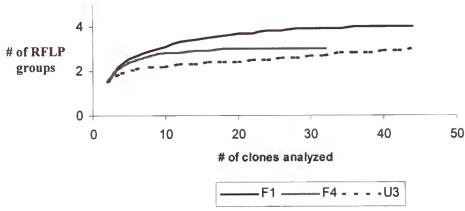

Figure 3-5. Rarefaction analysis for *Clostridium* Cluster IV clone libraries for soil samples from the Everglades.

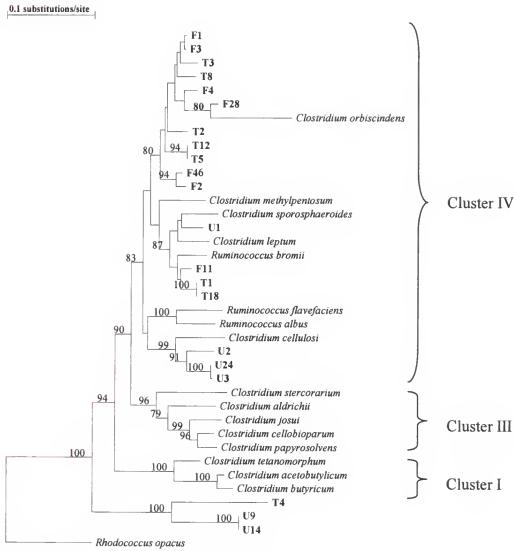

Figure 3-6. Phylogenetic tree of *Clostridium* Cluster IV 16S rRNA gene clone sequences obtained from Everglades soil (F1, F; F4, T; U3, U). Numbers at branch points refer to bootstrap analysis based on 100 resampling.

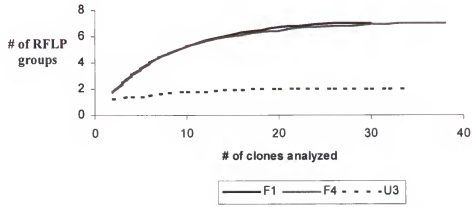

Figure 3-7. Rarefaction analysis for *Clostridium* Cluster XIV clone libraries for soil samples from the Everglades.

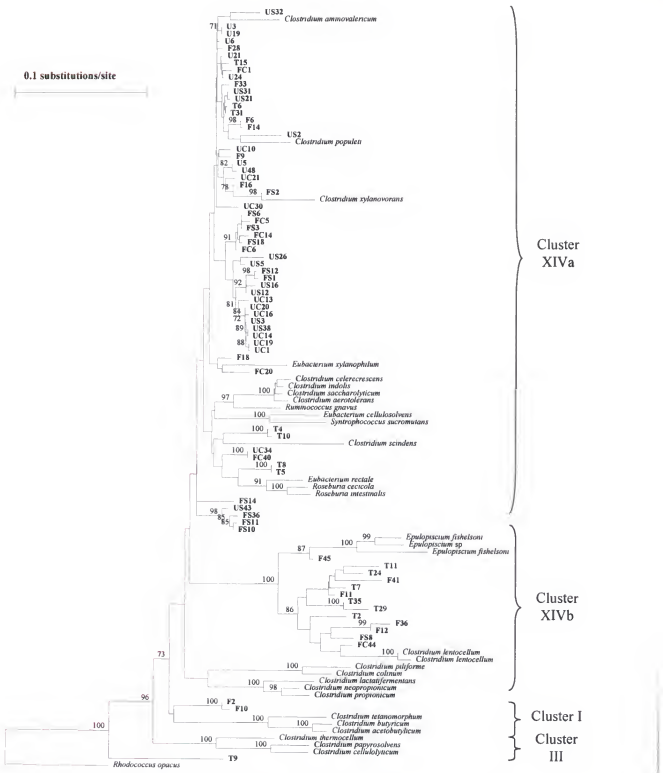

Figure 3-8. Phylogenetic tree of *Clostridium* Cluster XIV 16S rRNA gene clone sequences obtained from Everglades soils (F1, F; F4, T; U3, U; cattail, C; sawgrass, S). Numbers at branch points refer to bootstrap analysis based on 100 resampling.

CHAPTER 4
CHARACTERIZATION OF FERMENTATIVE PROCESSES IN BENTHIC PERIPHYTON MATS, WITH THE EMPHASIS ON CELLULOLYTIC AND FERMENTATIVE *Clostridium* COMMUNITIES

Previous chapters suggest that nutrient impact in wetlands leads to changes in fermentative and cellulolytic processes and composition of *Clostridium* assemblages. Moreover, data obtained from oligotrophic soil microcosms and plant material microcosms provided evidence that phosphorus may not be limiting for microorganisms in oligotrophic Everglades soils. This observation points to the importance of another natural organic source, such as benthic periphyton mat (floc), which is only seen in oligotrophic soils. Benthic periphyton mat may provide the major part of carbon needed for microbial communities in these soils. It is thought that the benthic periphyton mats are formed from senescing perphyton and macrophytes (Noe et al. 2003), and contains eukaryotic algae and bacteria. Algae perfom photosynthesis, providing substrate to bacteria, indicating that the benthic periphyton layer may have its own carbon cycling mechanisms.

There are limited numbers of studies specifically conducted on the benthic periphytonn mat. However, the majority of these studies focused almost exclusively on the role of benthic periphyton mats in nutrient cycling mechanisms in oligotrophic soils of the Everglades (Noe et al. 2003; D'Angelo and Reddy 1994a, 1994b). No report is available on the fermentative potential of the benthic periphyton and its *Clostridium* assemblage composition in the Everglades. In addition, data presented in Chapter 2 suggested that soil underlying the benthic mat is carbon limited, indicating the

importance of the benthic periphyton in the carbon cycling budget in oligotrophic Everglades soils. Therefore, the objective of this chapter is to investigate potential fermentation of various carbon sources (glucose and cellulose) in benthic periphyton mats and to observe relationships between other bacterial groups (fermentative, syntrophic and methanogenic) in microcosms. It is also this chapter's objective to investigate fermentative and cellulolytic *Clostridium* assemblage composition and to compare the results with data previously obtained from eutrophic, transition and oligotrophic soils.

Materials and Methods

Site Characteristics, Sampling and Biogeochemical Characterization

Soil cores were collected by South Florida Water Management District staff from the oligotrophic region of the Florida Everglades Water Conservation Area 2A (WCA2A) under flooded conditions in Fall, 2004. Site characteristics were previously described in Chapter 2. Benthic peripyhton layer was removed from core samples and composite subsamples to be used for microcosm experiments and enumeration were stored at 4°C until analysis (within 2 to 7 days after sampling), and subsamples intended for DNA analysis were stored at –70°C. Ammonium, total phosphorus, total inorganic phosphorus, extractable total organic carbon, and microbial organic carbon were determined by the Wetland Biogeochemistry Laboratory as described previously (Wright and Reddy 2001a; Castro et al. 2002; Chauhan et al. 2004).

Microbial Enumeration

The most probable number (MPN) technique with 5 replicates per dilution was used for enumeration studies. The MPN medium contained peptone (10 g/L), NaCl (5 g/L) and bromocresol purple (0.0085 g/L), cysteine-sodium sulfide (2% to provide final

redox potential of 110-200 mV). Glucose (20 mM) and cellulose powder were added to MPN tubes for fermentation bacteria and cellulolytic bacteria, respectively. For fermentative MPNs, color change from purple to yellow due to acidity was counted as positive. For cellulose MPNs, tubes changing color due to acidity and showing structural change in cellulose substrate were counted positive.

Microcosm Studies

Composite benthic periphyton mat samples (2 g, wet weight) were mixed with 50 mL basal carbonate yeast extract trypticase (BCYT) media (Touzel and Albagnac 1983) in 100 mL serum tubes. BCYT also included resazurin (1%), cysteine-sodium sulfide (2%) and a carbon source. Compounds used as carbon source include glucose (20 mM) and cellulose (0.162 g). All media, stock solutions and microcosms were prepared under nitrogen gas stream to provide anaerobic conditions. Vials were closed with robber stoppers and aluminum seals, and incubated at 28°C.

Fatty Acid and Methane Measurement

Liquid samples (1 mL) were collected weekly from microcosms. These samples were centrifuged, filtered through 0.2 μm filters, and stored at –20°C until analysis. Fatty acids were measured with high-pressure liquid chromatograph (HPLC) (Waters Corp., Milford, MA) equipped with a UV detector set at 210 nm. Aminex HP 87 H column (300 x 7.5 mm) was used with sulfuric acid (0.5 mM) as mobile phase at a flow rate of 0.6 mL/min. Methane formation in the head space was determined by Shimadzu 8A gas chromatograph equipped with a Carboxen 1000 column (Supelco, Bellefonte, PA) and flame ionization detector set at 110°C. Nitrogen was used as carrier gas and the oven temperature was 160°C. The pressure in the headspace was measured with a digital pressure device (DPI 705; Druck, New Fairfield, CT).

Extraction of DNA and PCR Amplification

DNA was extracted from benthic periphyton samples by using Power Soil DNA isolation kit (Mobio, Solana Beach, CA) according to manufacturer's instructions. After extraction, DNA was electrophoresed through 0.7 to 1% agarose gel in Tris-Acetate-EDTA (TAE) buffer. DNA was stored at –20°C until further analysis.

Primer names, sequences, annealing temperatures, and target groups for amplification by the polymerase chain reaction (PCR) are presented in Table 3-1. PCR reaction mixtures contained 10 µL of HotStarTaq master mix (Qiagen, Valencia, CA), 7 µL of distilled H_2O, 1 µL of each primer (10 pmol/µL) and 1 µL of diluted DNA solution. PCR cycling was performed at 94°C for 1 minute for denaturation and at 72°C for 1 minute for chain extension. Annealing was performed for 1 minute for *Clostridium*-specific primers at temperatures shown in Table 3-1. Reaction mixtures were subjected to 40 cycles for *Clostridium*-specific primers in a Perkin-Elmer Model 2400 Thermal Cycler (Perkin-Elmer, Norwalk, CT). An initial activation step of 95°C for 15 minutes was required for HotStarTaq master mix. An additional seven minutes were added for chain extension at the end of reactions.

Cloning of 16S rRNA Genes and RFLP Analysis

Fresh PCR products were ligated into pCRII-TOPO cloning vector (Invitrogen, Carlsbad, CA) and transformed into chemically competent *E. coli* cells (TOP10F') according to the vendor's instructions. Individual colonies were screened by direct PCR amplification and restriction fragment length polymorphism (RFLP) analysis was performed using digestion enzymes *Hha*I + *Eco*RV for Cluster I, *Alu*I for Cluster III, and *Msp*I for Cluster IV clones. Selection of digestion enzymes for RFLP was based on

in silico analysis of previously identified 16S rRNA genes of *Clostridium* species in National Center Biotechnology Information (NCBI) database using CloneMap software (version 2.11, CGC Scientific Inc, Ballwin, MO). Digestion reactions were analyzed in 2% agarose gels.

Sequencing and Phylogenetic Analysis

Selected clones representatives of different digestion patterns were sequenced by University of Florida's Interdisciplinary Center for Biotechnology Research core sequencing facility. Sequences were compared with previously identified sequences in NCBI database using BLAST (Altschul et al. 1990). The sequences obtained from samples were initially aligned with closely matched sequences from NCBI database and clone sequences obtained from soil samples using the Pileup function of GCG Package (Accelrys Inc., San Diego, CA) and adjusted manually with ClustalX version 1.8 (Thompson et al. 1997). Phylogenetic trees were generated with TREECON (Van de Peer and De Wachter 1994, 1997) using a neighbor-joining method. Bootstrap analysis was performed with 100 resamplings of the DNA sequences to estimate the confidence of the tree topology.

Results and Discussion

Biogeochemical Characterization

Biogeochemical parameters for phosphorus, carbon and nitrogen in benthic periphyton samples are presented in Tables 4-1 and 4-2. Benthic periphyton samples appeared to have lower total phosphorus content than do soils samples. Total inorganic phosphorus content of benthic layer was also found to be lower than F1, F4 and U3 soils. However, total carbon in benthic layer was higher than U3 soil and lower than U3 (ridge) soil. Benthic periphyton mats showed the lowest microbial biomass carbon with

relatively high standard deviation. On the other hand, highest extractable total organic carbon was observed in benthic periphyton samples. Total nitrogen in benthic layer samples was similar to F1 and F4 soils and slightly lower than U3 soil.

Enumeration of Cellulolytic and Fermentative Bacteria

No significant difference in MPNs of cellulolytic bacteria was observed between U3 and benthic periphyton mat (Tables 2-5 and 4-3). However, benthic periphyton and U3 cellulose MPNs were 10 fold lower that F1, F4 and U3 (ridge) MPNs. MPN of fermentation bacteria, however, were 100 fold higher in benthic layer than in F1, F4 and U3 soils, and 10 fold higher than in U3 (ridge) soil.

Carbon Cycling Potential

In terms of fatty acid production rate and behavior, benthic periphyton-control microcosms showed a trend similar to that observed in U3-control microcosms (Figures 4-1 and 2-3). However, in U3 microcosms, acetate was almost depleted when methane production was observed, whereas in benthic mat-glucose microcosms methane production did not correspond with acetate depletion, even though there was a fluctuation in acetate concentration between days 35 and 50. In addition, U3-control microcosms did not show any significant initial methane production until day 35, whereas benthic mat-control microcosms produce methane in small but increasing rate from the beginning of the experiment. Indigenous butyrate and propionate trends in benthic periphyton microcosms were found to be similar to those observed in U3 microcosms. In terms of methane, butyrate and propionate production and consumption trends, benthic mat-control microcosms were similar to U3 (ridge)-control microcosms. However, a sharp increase in methane production was observed earlier in U3 (ridge) microcosms than in benthic mat microcosms.

Carbon fractionations in different fermentation products in benthic periphyton microcosms spiked with glucose and cellulose at the end of the experiment is presented in Table 4-4. In glucose microcosms, benthic periphyton samples showed methane production and acetate depletion (Figure 4-2 A). This trend is contrary to those observed in U3 and U3 (ridge) microcosms with glucose (Figure 2-5); these microcosms did not produce methane, and acetate was not consumed. Moreover, in benthic periphyton microcosms, butyrate accumulated in lower concentration and appeared to be partially consumed at the end of the experiment whereas U3 and U3 (ridge) microcosms accumulated butyrate in higher concentration and no butyrate was consumed. This suggests that benthic periphyton has higher syntrophic potential than does the underlying soil. Indeed, it was observed that butyrate-oxidizing bacteria are at least 10 times higher in benthic periphyton layer than in U3 soil. Similarly, 10 times more hydrogen-utilizing bacteria were detected in benthic periphyton samples (Chauhan et al., unpublished data). When compared with glucose microcosms with F1 and F4 soils, benthic mat microcosms exhibited longer lag period before showing any significant methane production.

Cellulose microcosms with benthic periphyton samples also showed significantly different fermentation and methanogenesis patterns (Figure 4-2 B) compared to cellulose microcosms with U3 and U3 (ridge) soils (Figure 2-7). Acetate accumulation was greater in benthic mat-cellulose microcosms, and depletion of acetate coincided with a sharp increase in methane formation, which began later in these microcosms than in U3 microcosms. Initial methane formation from the beginning of the experiment was observed. Considering the fact that benthic periphyton layer has higher numbers of hydrogenotrophic bacteria than does U3 soil, it is likely that the initial methane formation

in benthic mat-cellulose microcosm is a result of hydrogenotrophic methanogenesis. However, methane formation by hydrogenotrophic methanogens was lower than expected, possibly due to activities of homoacetogenic bacteria in these microcosms.

Interestingly, the same phenomenon in which methane production halted for a period of time was also detected in benthic mat-cellulose microcosm at day 20. Possible explanations were discussed regarding this phenomenon in detail in Chapter 2. Briefly, the phenomenon may be attributed to sulfate mediated anaerobic methane oxidation (Martens and Berner 1977; Valentine 2002; Megonigal et al. 2004). However, sulfate additions in 5 mM concentration to benthic mat-cellulose microcosms in the late stage of the experiment did not reveal conclusive evidence supporting this possibility. Theoretically, other alternative electron acceptors can be utilized during the methane oxidation. However, there has been no report providing evidence to support this theory (Valentine 2002). Cellulose appeared to promote propionate production in benthic periphyton microcosms. However, no propionate consumption was observed. Similar trend was also observed in U3 microcosms with cellulose.

Phylogenetic Analysis of *Clostridium* 16S rRNA Gene Sequences from Floc Layer

RFLP digestion of *Clostridium* Cluster I sequences revealed the presence of 5 RFLP patterns in benthic periphyton samples and rarefaction curve for Cluster I clones approached the plateau of a complete clone library, indicating all possible phylotypes were recovered (Figure 4-3). The majority of the patterns that were commonly detected in soil samples were also observed in benthic mat samples. Phylogenetic analysis of Cluster I sequences from benthic mat samples, previously obtained sequences from soil samples, and previously identified sequences from NCBI database revealed the phylogenetic tree presented in Figure 4-4. Addition of benthic periphyton sequences did not change the

general branching, and the novel branch that was observed previously remain the same. Only one clone from benthic periphyton samples, FLOC8, was located in this branch. Similar to Cluster I clone sequences from soil samples, majority of benthic mat sequences showed less than 98% similarity to known Cluster I species. Clone FLOC19 was 99% similar to *C. bowmanii*, a psychrophilic and saccharolytic species (Spring et al. 2003). Clones FLOC21, FLOC3, and FLOC46 grouped with *C. saccharoperbutylacetonicum*, and show 99% similarity to this species. *Clostridium saccharoperbutylacetonicum* is a well-known solvent producing species and these clones that group with this species may perform similarly. In the same branch, clone FLOC18 grouped with *C. butylicum*, the type species of genus *Clostridium*, with 99% sequence similarity. Clones FLOC45 and FLOC33 were found to be 98% similar to *C. quinii* and FLOC34 was found to be close relative of *C.favososporum* with 99% similarity. In general, benthic periphyton clone sequences show similar distributions with soil clones sequences. The only exception is clone FLOC8, which was placed in the novel branch. The single benthic periphyton clone in the novel branch may suggest the presence of this species in benthic mat but also suggests that the species belonging to the novel branch is present in low number in benthic periphyton.

Rarefaction analysis of Cluster III clone sequences indicated that all possible phylotypes were represented (Figure 4-5). It was also observed that the benthic periphyton mat has similar diversity with U3 soil. Phylogenetic tree of *Clostridium* Cluster III with benthic periphyton clone sequences did not provide any significant difference in terms of specific branching in the tree (Figure 4-6). Clone FLOC5 was found to be 98% similar to *C. cellobioparum* and *C. termitidis*, and clone FLOC13 was

98% similar to *Bacteroides cellulosolvens*. All other clone sequences from benthic periphyton samples were less than 98% similar to any known Cluster III sequences. Benthic mat clone sequences were distributed with soil clone sequences. This may indicate that *Clostridium* Cluster III community, which is a cellulolytic community, in benthic mat, does not differ from that in soils.

RFLP digestion of Cluster IV clone sequences revealed that benthic layer has higher degree of diversity than soils samples (Figure 4-7). Phylogenetic tree of *Clostridium* Cluster IV produced a branching separating known cellulolytic and noncellulolytic species (Figure 4-8). The branch containing *Ruminococcus flavefaciens*, *R. albus*, and *C. cellulosi* represent the cellulolytic branch of Cluster IV, and clones FLOC39, FLOC30 and FLOC5 were placed in the branch with clones from U3. None of the clones from eutrophic and transition soils clustered in this branch. This may suggest that Cluster IV species are affected by eutrophication.

Perhaps the most striking observation is that DNA isolated from benthic periphyton samples did not yield amplification product with primers specific for *Clostridium* Cluster XIV species. Cluster XIV, especially subcluster XVIa, contains various cellulose and hemicellulose degrading species. Absence of this cluster may be explained with lower cellulose and hemicellulose contents of benthic periphyton mat. This explanation is supported by previously published reports indicating importance of Cluster XIV species in environments such as rice paddy soils, landfills, and human intestines where plant material or cellulose is present (Hengstmann et al. 1999; Weber et al. 2001; Van Dyke and McCarthy 2002; Hayashi et al. 2002). However, it should also be noted that Cluster XIV includes species that can also utilize other components such as sugars and amino

acids. In a study conducted in a methanogenic landfill leachate bioreactor, Cluster XIVa species were associated with glucose fermentation but not cellulose degradation (Burrell et al. 2004).

Table 4-1. Moisture and phosphorus parameters for benthic periphyton samples from the oligotrophic region of the Everglades. Provided by the Wetland Biogeochemistry Laboratory.

Sampling Site	Moisture content (%)	Total phosphorus (mg/kg)[b]	Total Inorganic Phosphorus (mg/kg)
Floc	91 (2.0)[a]	354 (19)	141 (14)

[a] Standard deviations are presented in parentheses.
[b] Concentrations of different substances are expressed per kg (dry weight) of soil.

Table 4-2. Carbon and nitrogen parameters for benthic periphyton samples from the oligotrophic region of the Everglades. Provided by the Wetland Biogeochemistry Laboratory.

Sampling Site	Total Carbon (g/kg)	Microbial Biomass Carbon (mg/kg)[b]	Extractable Total Organic Carbon (mg/kg)	Total Nitrogen (g/kg)	Extractable Ammonium (mg/kg)
Floc	360.0 (40)[a]	1689 (1046)	3002 (469)	24.1 (3.9)	114 (25)

[a] Standard deviations are presented in parentheses.
[b] Concentrations of different substances are expressed per kg (dry weight) of soil.

Table 4-3. Most probable numbers of cellulolytic and fermentative bacteria in benthic periphyton samples

Sample	Cellulose	Fermentation
Floc	$3.2x10^4$ (1.08-9.2)[a]	$7.9x10^8$ (2.5-18.9)

[a] Confidence levels (95%) are presented in parentheses.

Table 4-4. Carbon fractionation in various fermentation products in benthic periphyton microcosms spiked with glucose and cellulose

	mmole Carbon	
	Glucose	Cellulose
Acetate	0.90	0.30
Butyrate	0.62	0.15
Propionate	0.20	0.94
Methane	0.67	0.91
Total C counted	2.39	2.30
Carbon Unaccounted	3.61	3.94

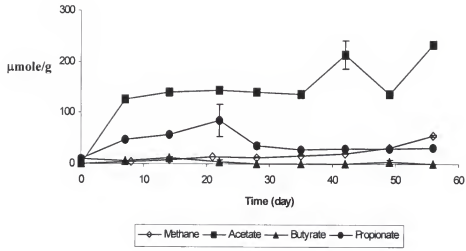

Figure 4-1. Acetate, butyrate, propionate and methane production potentials of benthic periphyton in microcosms containing no external carbon source. Error bars represent standard errors based on 5 replicates.

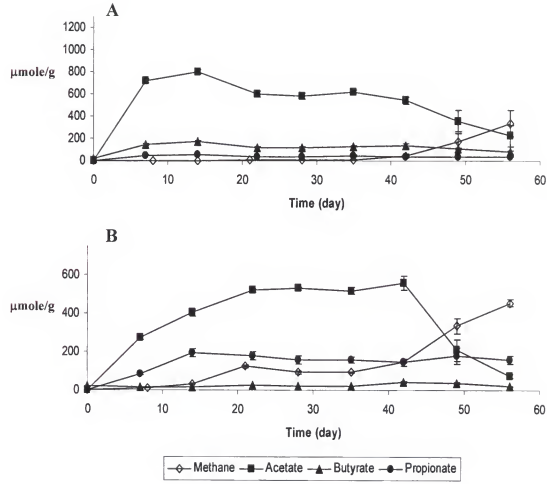

Figure 4-2 Effect of different carbon substrates on fermentation products and methane production in benthic periphyton. Error bars represent standard errors based on 5 replicates. A) Glucose microcosms. B) Cellulose microcosms.

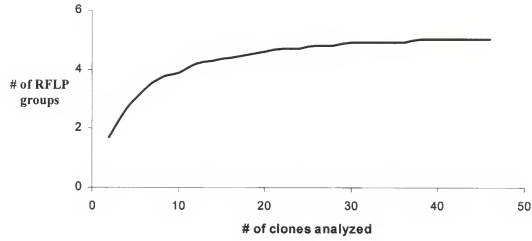

Figure 4-3. Rarefaction analysis for Cluster I clone libraries for benthic periphyton samples

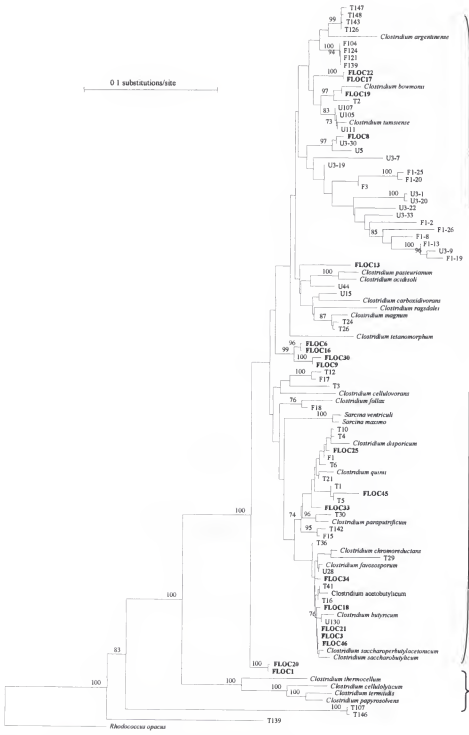

Figure 4-4. Phylogenetic tree of *Clostridium* Cluster I 16S rRNA gene clone sequences obtained from benthic periphyton samples. Clone sequences obtained from F1 (F), F4 (T) and U3 (U) soil samples were also included. Numbers at branch points refer to bootstrap analysis based on 100 resampling.

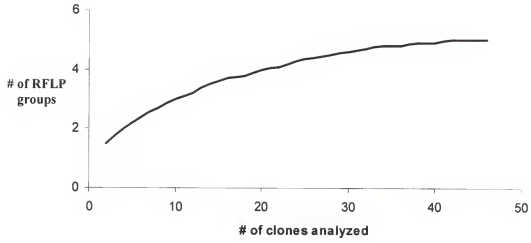

Figure 4-5. Rarefaction analysis for Cluster III clone libraries for benthic periphyton samples

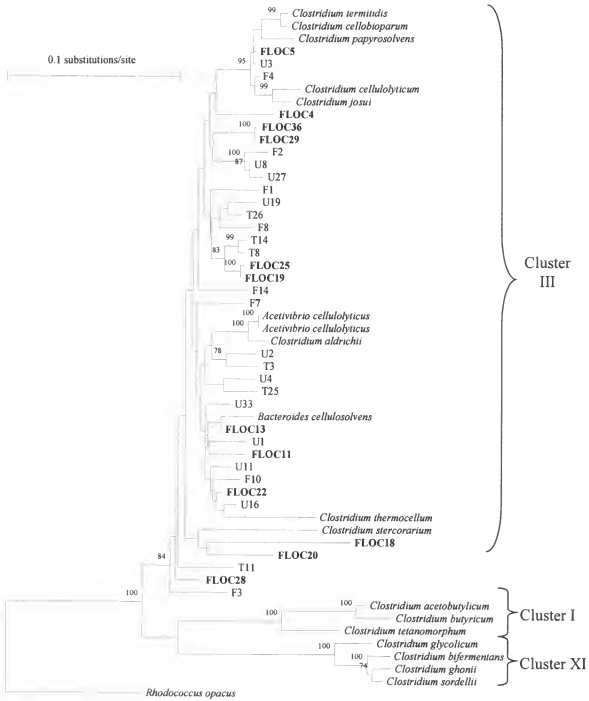

Figure 4-6. Phylogenetic tree of *Clostridium* Cluster III 16S rRNA gene clone sequences obtained from benthic periphyton samples. Clone sequences obtained from F1 (F), F4 (T) and U3 (U) soil samples were also included. Numbers at branch points refer to bootstrap analysis based on 100 resampling.

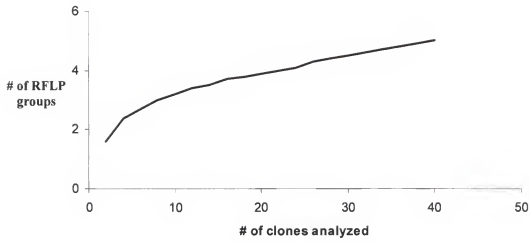

Figure 4-7. Rarefaction analysis for Cluster IV clone libraries for benthic periphyton samples

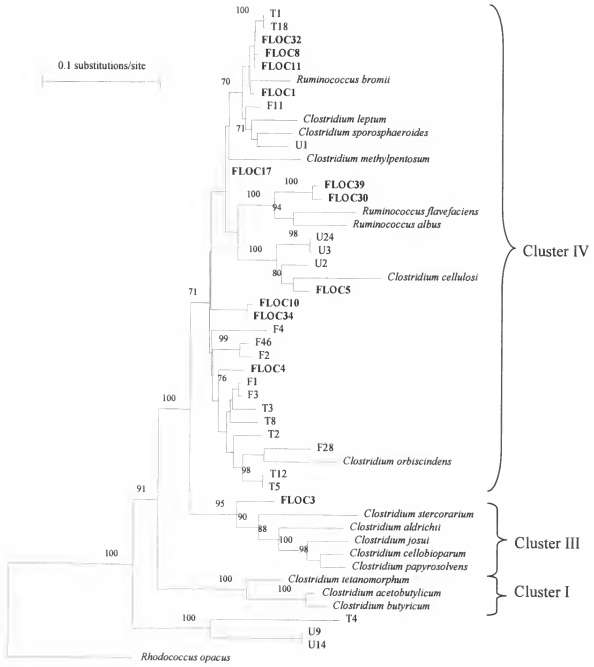

Figure 4-8. Phylogenetic tree of *Clostridium* Cluster IV 16S rRNA gene clone sequences obtained from benthic periphyton samples. Clone sequences obtained from F1 (F), F4 (T) and U3 (U) soil samples were also included. Numbers at branch points refer to bootstrap analysis based on 100 resampling.

CHAPTER 5
SUMMARY AND CONCLUSIONS

In anaerobic environments such as wetlands, microbial processes and microbial community structure is a function of biogeochemical characteristics and substrates such as soils and detrital material. Two of the major microbial groups present in wetlands are cellulolytic and fermentation species that provide carbon to higher trophic levels. It is likely that any biogeochemical change in these environments may directly or indirectly affect these groups, hence affecting the entire microbial food web. Similarly, any change in higher trophic organisms due to biogeochemical parameters may have direct effects on other groups, such as those responsible for cellulose degradation and fermentation. The Everglades is a good ecosystem for studying their effects due to the fact that effect of various characteristics of this system on cellulolytic and fermentative bacteria can be investigated in the same time. Therefore, cellulose degradation and fermentation must be investigated to understand true nature of the Everglades and impact of nutrient loading on carbon cycling mechanism.

This research was conducted to understand the role of microbial groups on cellulolytic and fermentative processes, the first step in anaerobic terminal carbon mineralization. The overall goal was to draw a more complete picture of nutrient impacts on carbon cycling, including establishing linkages between carbon input and microbial community structure and function. The study was conducted with soil and benthic periphyton samples collected from three different sites in the Everglades Water Conservation Area 2A, showing different levels of eutrophication.

We investigated potential fermentation of various carbon sources including glucose, cellulose, and plant material in Everglades soils toward understanding the effect of nutrient impact on the carbon mineralization and relationship between bacterial groups. MPN enumeration showed that cellulolytic bacterial numbers in eutrophic, transition and oligotrophic ridge soils were 10 fold higher than in oligotrophic slough area soils. However, no significant difference was observed in terms of MPN of fermentative bacteria between soils samples. Microcosm experiments indicated that carbon cycling mechanism is more active in eutrophic soils than in oligotrophic soils. In microcosm experiments containing glucose showed that F1 and F4 soils posses microbial community or well established microbial association that can eliminate excess fatty acids or other by-products. MPN enumeration of fermentative bacteria, total consumption of glucose and acetate production trend in the first seven days by F1 and U3 soils indicated similar activities of fermentative bacteria in the two soils. These observations emphasized the importance of syntrophic activity (synthrophs and hydrogenotrophic methanogens) on fermentation product pattern. This conclusion was supported by a recently published report on Everglades soils. In the Everglades, plant material type as carbon and nutrient source appeared to be significant only in F1 soils and it seemed that cattail slightly inhibited methanogenesis possibly due to presence of higher phenolic compounds. However, MPN of fermentative bacteria indicated that cattail has greater impact, possibly because of higher neutral detergent soluble fraction content containing sugar, pectin, proteins, etc. Higher TP content of cattail did not provide any significant difference, suggesting that phosphorus is not a determining factor for methanogenesis in U3 soils. This conclusion was supported by a microcosm experiment containing U3 soils and water

amended with different amounts of phosphorus. No significant difference was observed in methane formation. However, addition of carbon sources to these microcosms resulted in immediate increase in methane formation. These observations confirm that phosphorus is not the first priority for microorganisms in oligotrophic soils of the Everglades.

We also investigated and compared cellulolytic and fermentative *Clostridium* community by molecular techniques targeting the 16S rRNA gene. Clone sequences belonging to different *Clostridium* clusters were obtained and analyzed with phylogenetic techniques. These techniques revealed a novel branch in *Clostridium* Cluster I tree and included clones only from F1 and U3 soils. Absence of sequences from F4 in this novel branch may be due to unbalanced carbon cycling, adjusting itself to the new nutrient rich status. Phylogenetic analysis of *Clostridium* Cluster XIV sequences also revealed a significant difference based on origin of soil samples. No clone sequence from U3 soil samples were placed in Cluster XIVb branch. Sequences obtained from plant microcosms confirmed this observation. Presence of Cluster XIVa sequences in all soil samples indicated importance of these cluster considering the fact that Cluster XIVa species are, in general, specialized in cellulose and hemicellulose degradation. T-RFLP analysis using Cluster XIV primers with soil samples collected from F1, F4 and U3 regions in various time statistically detected the difference between soils. It should also be noted that RFLP patterns obtained with digestion enzymes did not completely corresponded with phylogenetic analysis. Short PCR products and high sequence similarity between species in each cluster renders the true usefulness of RFLP pattern anaylsis for *Clostridium* species. However, since Cluster XIV species are divided into 2 subclusters due to

significant 16S rRNA sequence difference, T-RFLP can be a useful tool for monitoring effects of eutrophication in the Everglades.

In Chapter 4, we focused solely on cellulolytic and fermentative characterization of benthic periphyton that is only found in oligotrophic regions of the Everglades. Microcosm containing benthic periphyton samples showed that benthic periphyton has similar or slightly higher carbon cycling potential compared to U3 soils. However, glucose microcosm with benthic periphyton samples produced methane, whereas U3 soil microcosm did not. This suggests that syntrophic and methanogenic bacteria are more active in benthic periphyton samples than in U3 soils. Especially, cellulose microcosms appear to promote propionate production in benthic periphyton microcosms. However, absence of propionate consumption may be an indication of absence of syntrophic bacteria utilizing propionate. Most probable number of fermentative bacteria was found to be 100 times higher in benthic periphyton than in F1, F4 and U3 soils. This clearly indicates that benthic periphyton is an ideal environment for fermentative bacteria. On the other hand, most probable number of cellulolytic bacteria was not significantly different than U3 soils. The phylogenetic tree of *Clostridium* Cluster I with sequences from benthic periphyton and soil samples revealed the same novel branch that was seen in Chapter 3. Only one benthic periphyton clone sequence was placed in this branch. It is possible to speculate that species belonging to the novel branch are present in the benthic periphyton, even though in low number. In phylogenetic tree of Cluster IV, floc sequences grouped with cellulolytic members of this cluster. This grouping was observed with sequences from U3 soils. This may be an indication that U3 soils and benthic periphyton, but not F1 and F4 soils, select for celluloytic bacteria belonging to

Cluster IV. It was not possible to generalize this speculation for all cellulolytic species because phylogenetic tree of Cluster III, a cellulolytic cluster, did not show any selection based on origin of samples. The most striking observation on benthic periphyton samples was that no PCR amplification was obtained with primers specific to *Clostridium* Cluster XIV. Cluster XIV species are not only associated with cellulolytic and hemicellulolytic activities but also fermentative functions in various environments. These findings indicated that microbial composition and functions in benthic periphyton differ from underlying soil.

Clearly, the effect of eutrophication was observed on carbon cycling mechanism with the emphasis on cellulolytic and fermentative processes and microorganisms in the Everglades. The present study contributed to a greater understanding of carbon cycling mechanism in wetland environments, and provided molecular databases that future studies may rely on.

LIST OF REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403-410.

Angert, E. R. and Clements, K. D. (2004) Initiation of intracellular offspring in *Epulopiscium*. Mol. Microbiol. 51, 827-835.

Angert, E. R., Clements, K. D. and Pace, N. R. (1993) The largest bacterium. Nature 362, 239-241.

Barcenilla, A., Pryde, S. E., Martin, J. C., Duncan, S. H., Stewart, C. S., Henderson, C. and Flint, H. J. (2000) Phylogenetic relationships of butyrate-producing bacteria from the human gut. Appl. Environ. Microbiol. 66, 1654-1661.

Beguin, P. and Lemaire, M. (1996) The cellulosome: An exocellular, multiprotein complex specialized in cellulose degradation. Crit. Rev. Biochem. Mol. Biol. 31, 201-236.

Berkhoff, H. A. (1985) *Clostridium colinum* sp. nov., nom. rev., the causative agent of ulcerative enteritis (quail disease) in quail, chickens, and pheasants. Int. J. Syst. Bact. 35, 155-159.

Borsodi, A. K., Vladar, P., Cech, G., Gedeon, G., Beszteri, B., Micsinai, A., Reskone, M. N. and Marialigeti, K. (2003) Bacterial activities in the sediment of Lake Velencei, Hungary. Hydrobiologia 506, 721-728.

Burrell, P. C., O'Sullivan, C., Song, H., Clarke, W. P. and Blackall, L. L. (2004) Identification, detection, and spatial resolution of *Clostridium* populations responsible for cellulose degradation in a methanogenic landfill leachate bioreactor. Appl. Environ. Microbiol. 70, 2414-2419.

Cardon, B. P. and Barker, H. A. (1946) Two new amino-acid-fermenting bacteria, *Clostridium propionicum* and *Diplococcus glycinophilus*. J. Bacteriol. 52, 629-634.

Castro, H., Ogram, A. and Reddy, K. R. (2004) Phylogenetic characterization of methanogenic assemblages in eutrophic and oligotrophic areas of the Florida Everglades. Appl. Environ. Microbiol. 70, 6559-6568.

Castro, H., Reddy, K. R. and Ogram, A. (2002) Composition and function of sulfate-reducing prokaryotes in eutrophic and pristine areas of the Florida Everglades. Appl. Environ. Microbiol. 68, 6129-6137.

Chamkha, M., Garcia, J. L. and Labat, M. (2001) Metabolism of cinnamic acids by some *Clostridiales* and emendation of the descriptions of *Clostridium aerotolerans*, *Clostridium celerecrescens* and *Clostridium xylanolyticum*. Int. J. Syst. Evol. Microbiol. 51, 2105-2111.

Chauhan, A., Ogram, A. and Reddy, K. R. (2004) Syntrophic-methanogenic associations along a nutrient gradient in the Florida Everglades. Appl. Environ. Microbiol. 70, 3475-3484.

Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J. J., Fernandezgarayzabal, J., Garcia, P., Cai, J., Hippe, H. and Farrow, J. A. E. (1994) The phylogeny of the genus *Clostridium*: Proposal of 5 new genera and 11 new species combinations. Int. J. Syst. Bact. 44, 812-826.

Conrad, R. (1999) Contribution of hydrogen to methane production and control of hydrogen concentrations in methanogenic soils and sediments. FEMS Microbiol. Ecol. 28, 193-202.

Conrad, R. and Klose, M. (1999) Anaerobic conversion of carbon dioxide to methane, acetate and propionate on washed rice roots. FEMS Microbiol. Ecol. 30, 147-155.

Coughlan, M. P. and Mayer, F. (1991) The cellulose-decomposing bacteria and their enzyme systems. In The Prokaryotes: A handbook on the Biology of Bacteria, ed. A. Balows, H. G. Truper, M. Dworkin, W. Harder and K. H. Schleifer, Vol. 1, pp. 460-516. Springer-Verlag, New York.

D'Angelo, E. M. and Reddy, K. R. (1994a) Diagenesis of organic-matter in a wetland receiving hypereutrophic lake water: 2. Role of inorganic electron acceptors in nutrient release. J. Environ. Qual. 23, 937-943.

D'Angelo, E. M. and Reddy, K. R. (1994b) Diagenesis of organic-matter in a wetland receiving hypereutrophic lake water: 1. Distribution of dissolved nutrients in the soil and water column. J. Environ. Qual. 23, 928-936.

DeBusk, W. F. and Reddy, K. R. (1998) Turnover of detrital organic carbon in a nutrient-impacted Everglades marsh. Soil Sci. Soc. Am. J. 62, 1460-1468.

DeForest, J. L., Zak, D. R., Pregitzer, K. S. and Burton, A. J. (2004) Atmospheric nitrate deposition and the microbial degradation of cellobiose and vanillin in a northern hardwood forest. Soil Biol. Biochem. 36, 965-971.

Drake, H. L., Aumen, N. G., Kuhner, C., Wagner, C., Griesshammer, A. and Schmittroth, M. (1996) Anaerobic microflora of Everglades sediments: Effects of nutrients on population profiles and activities. Appl. Environ. Microbiol. 62, 486-493.

Duncan, S. H., Hold, G. L., Barcenilla, A., Stewart, C. S. and Flint, H. J. (2002) *Roseburia intestinalis* sp. nov., a novel saccharolytic, butyrate-producing bacterium from human faeces. Int. J. Syst. Evol. Microbiol. 52, 1615-1620.

Entry, J. A. (2000) Influence of nitrogen on cellulose and lignin mineralization in blackwater and redwater forested wetland soils. Biol. Fert. Soils 31, 436-440.

Eriksson, K. E. L., Blanchette, R. A. and Ander, P. (1990) Microbial and enzymatic degradation of wood and wood components. Springer-Verlag, Germany.

Franks, A. H., Harmsen, H. J. M., Raangs, G. C., Jansen, G. J., Schut, F. and Welling, G. W. (1998) Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA targeted oligonucleotide probes. Appl. Environ. Microbiol. 64, 3336-3345.

Freeman, C., Liska, G., Ostle, N. J., Lock, M. A., Reynolds, B. and Hudson, J. (1996) Microbial activity and enzymic decomposition processes following peatland water table drawdown. Plant Soil 180, 121-127.

Freeman, C., Ostle, N. and Kang, H. (2001) An enzymic 'latch' on a global carbon store: A shortage of oxygen locks up carbon in peatlands by restraining a single enzyme. Nature 409, 149.

Fukuzaki, S., Nishio, N., Shobayashi, M. and Nagai, S. (1990) Inhibition of the fermentation of propionate to methane by hydrogen, acetate, and propionate. Appl. Environ. Microbiol. 56, 719-723.

Garcia, J. L., Patel, B. K. C. and Ollivier, B. (2000) Taxonomic phylogenetic and ecological diversity of methanogenic Archaea. Anaerobe 6, 205-226.

Hayashi, H., Sakamoto, M. and Benno, Y. (2002) Fecal microbial diversity in a strict vegetarian as determined by molecular analysis and cultivation. Microbiol. Immunol. 46, 819-831.

Hengstmann, U., Chin, K. J., Janssen, P. H. and Liesack, W. (1999) Comparative phylogenetic assignment of environmental sequences of genes encoding 16S rRNA and numerically abundant culturable bacteria from an anoxic rice paddy soil. Appl. Environ. Microbiol. 65, 5050-5058.

Janssen, P. H. (1991) Isolation of *Clostridium propionicum* strain 19acry3 and further characteristics of the species. Arch. Microbiol. 155, 566-571.

Jones, D. T. and Woods, D. R. (1986) Acetone-butanol fermentation revisited. Microbiol. Rev. 50, 484-524.

Karnholz, A., Kusel, K., Gossner, A., Schramm, A. and Drake, H. L. (2002) Tolerance and metabolic response of acetogenic bacteria toward oxygen. Appl. Environ. Microbiol. 68, 1005-1009.

Kochrose, M. S., Reddy, K. R. and Chanton, J. P. (1994) Factors controlling seasonal nutrient profiles in a subtropical peatland of the Florida Everglades. J. Environ. Qual. 23, 526-533.

Koike, S. and Kobayashi, Y. (2001) Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococus albus* and *Ruminococcus flavefaciens*. FEMS Microbiol. Lett. 204, 361-366.

Lane, D. J. (1991) 16S/23S rRNA sequencing. In Nucleic acid techniques in bacterial systematics, ed. E. Stackebrandt and M. Goodfellow, pp. 115-175. John Wiley & Sons, Inc., New York, N.Y.

Lehmann-Richter, S., Grosskopf, R., Liesack, W., Frenzel, P. and Conrad, R. (1999) Methanogenic archaea and CO_2-dependent methanogenesis on washed rice roots. Environ. Microbiol. 1, 159-166.

Leschine, S. B. (1995) Cellulose degradation in anaerobic environments. Annu. Rev. Microbiol. 49, 399-426.

Lin, C. Z. and Stahl, D. A. (1995) Taxon-specific probes for the cellulolytic genus *Fibrobacter* reveal abundant and novel equine-associated populations. Appl. Environ. Microbiol. 61, 1348-1351.

Lynd, L. R., Weimer, P. J., van Zyl, W. H. and Pretorius, I. S. (2002) Microbial cellulose utilization: Fundamentals and biotechnology. Microbiol. Mol. Biol. Rev. 66, 506-577.

Martens, C. S. and Berner, R. A. (1977) Interstitial water chemistry of anoxic long island sound sediments .1. Dissolved-gases. Limnol. Oceanogr. 22, 10-25.

Mechichi, T., Labat, M., Garcia, J. L., Thomas, P. and Patel, B. K. C. (1999) Characterization of a new xylanolytic bacterium, *Clostridium xylanovorans* sp. nov. Syst. Appl. Microbiol. 22, 366-371.

Megonigal, J. P., Hines, M. E. and Visscher, P. T. (2004) Anaerobic metabolism: Linkages to trace gases and aerobic processes. In Biogeochemistry, ed W. H. Schlesinger, pp. 317-424. Elsevier-Pergamon, Oxford,UK.

Miller, T. L. and Wolin, M. J. (1995) Bioconversion of cellulose to acetate with pure cultures of *Ruminococcus albus* and a hydrogen-using acetogen. Appl. Environ. Microbiol. 61, 3832-3835.

Murray, W. D., Hofmann, L., Campbell, N. L. and Madden, R. H. (1986) *Clostridium lentocellum* sp. nov., a cellulolytic species from river sediment containing paper-mill waste. Syst. Appl. Microbiol. 8, 181-184.

Newman, S., Kumpf, H., Laing, J. A. and Kennedy, W. C. (2001) Decomposition responses to phosphorus enrichment in an Everglades (USA) slough. Biogeochemistry 54, 229-250.

Noe, G. B., Scinto, L. J., Taylor, J., Childers, D. L. and Jones, R. D. (2003) Phosphorus cycling and partitioning in an oligotrophic Everglades wetland ecosystem: A radioisotope tracing study. Freshwater Biol. 48, 1993-2008.

Osborn, A. M., Moore, E. R. B. and Timmis, K. N. (2000) An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. Environ. Microbiol. 2, 39-50.

Palop, M. L., Valles, S., Pinaga, F. and Flors, A. (1989) Isolation and characterization of an anaerobic, celluloytic bacterium, *Clostridium celerecrescens* sp. nov. Int. J. Syst. Bact. 39, 68-71.

Palop, M. L., Valles, S., Pinaga, F. and Flors, A. (1991) Characterization of cellulase and xylanase activities of *Clostridium celerecrescens*. J. Chem. Technol. Biotechnol. 51, 105-114.

Prenger, J. P. and Reddy, K. R. (2004) Microbial enzyme activities in a freshwater marsh after cessation of nutrient loading. Soil Sci. Soc. Am. J. 68, 1796-1804.

Pryde, S. E., Duncan, S. H., Hold, G. L., Stewart, C. S. and Flint, H. J. (2002) The microbiology of butyrate formation in the human colon. FEMS Microbiol. Lett. 217, 133-139.

Rainey, F. A. and Janssen, P. H. (1995) Phylogenetic analysis by 16s ribosomal DNA sequence comparison reveals 2 unrelated groups of species within the genus *Ruminococcus*. FEMS Microbiol. Lett. 129, 69-73.

Reddy, K. R., Delaune, R. D., DeBusk, W. F. and Koch, M. S. (1993) Long-term nutrient accumulation rates in the Everglades. Soil Sci. Soc. Am. J. 57, 1147-1155.

Robert, C., Del'Homme, C. and Bernalier-Donadille, A. (2001) Interspecies H_2 transfer in cellulose degradation between fibrolytic bacteria and H_2-utilizing microorganisms from the human colon. FEMS Microbiol. Lett. 205, 209-214.

Schink, B. (1991) Syntrophism among Prokaryotes. In The Prokaryotes: A handbook on the biology of bacteria, ed. A. Balows, H. G. Truper, M. Dworkin, W. Harder and K. H. Schleifer, Vol. 1, pp. 276-299. Springer-Verlag, New York.

Schink, B. (1997) Energetics of syntrophic cooperation in methanogenic degradation. Microbiol. Mol. Biol. Rev. 61, 262-280.

Schoefer, L., Mohan, R., Schwiertz, A., Braune, A. and Blaut, M. (2003) Anaerobic degradation of flavonoids by *Clostridium orbiscindens*. Appl. Environ. Microbiol. 69, 5849-5854.

Schwarz, W. H. (2001) The cellulosome and cellulose degradation by anaerobic bacteria. Appl. Microbiol. Biotechnol. 56, 634-649.

Sharma, K. (2002) Microbial Cr(IV) reduction: Role of electron donors, acceptors, and mechanisms, with special emphasis on *Clostridium* spp. University of Florida. Ref Type: Thesis/Dissertation

Sleat, R. and Mah, R. A. (1985) *Clostridium populeti* sp. nov., a cellulolytic species from a woody-biomass digester. Int. J. Syst. Bact. 35, 160-163.

Smouter, H., Simpson, R. J. and Pearce, G. R. (1995) Water-soluble carbohydrates and *in vitro* digestibility of annual ryegrass (lolium-rigidum gaudin) sown at varying densities. Aust. J. Agr. Res. 46, 611-625.

Spring, S., Merkhoffer, B., Weiss, N., Kroppenstedt, R. M., Hippe, H. and Stackebrandt, E. (2003) Characterization of novel psychrophilic *Clostridia* from an Antarctic microbial mat: Description of *Clostridium frigoris* sp. nov., *Clostridium lacusfryxellense* sp. nov., *Clostridium bowmanii* sp. nov. and *Clostridium psychrophilum* sp. nov. and reclassification of *Clostridium laramiense* as *Clostridium estertheticum* subsp. laramiense subsp. nov. Int. J. Syst. Evol. Microbiol. 53, 1019-1029.

Stackebrandt, E. and Hippe, H. (2001) Taxonomy and Systematic. In *Clostridia*: Biotechnology and Medical Applications, ed. H. Bahl and P. Durre, pp. 19-48. Wiley-VCH Verlag GmbH, Weinheim, Germany.

Suen, J. C., Hatheway, C. L., Steigerwalt, A. G. and Brenner, D. J. (1988) *Clostridium argentinense* sp. nov., a genetically homogeneous group composed of all strains of *Clostridium botulinum* toxin type g and some nontoxigenic strains previously identified as *Clostridium subterminale* or *Clostridium hastiforme*. Int. J. Syst. Bact. 38, 375-381.

Thabet, O. B., Fardeau, M. L., Joulian, C., Thomas, P., Hamdi, M., Garcia, J. L. and Ollivier, B. (2004) *Clostridium tunisiense* sp. nov., a new proteolytic, sulfur-reducing bacterium isolated from an olive mill wastewater contaminated by phosphogypse. Anaerobe 10, 185-190.

Tholozan, J. L., Touzel, J. P., Samain, E., Grivet, J. P., Prensier, G. and Albagnac, G. (1992) *Clostridium neopropionicum* sp. nov., a strict anaerobic bacterium fermenting ethanol to propionate through acrylate pathway. Arch. Microbiol. 157, 249-257.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. (1997) The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876-4882.

Touzel, J. P. and Albagnac, G. (1983) Isolation and characterization of *Methanococcus mazei* strain mc3. FEMS Microbiol. Lett. 16, 241-245.

Uffen, R. L. (1997) Xylan degradation: A glimpse at microbial diversity. J. Ind. Microbiol. Biotechnol. 19, 1-6.

Valentine, D. L. (2002) Biogeochemistry and microbial ecology of methane oxidation in anoxic environments: a review. Antonie Van Leeuwenhoek Int. J. Gen. Mol. Microbiol. 81, 271-282.

Van de Peer, Y. and De Wachter, R. (1994) TREECON for windows, a software package for the construction and drawing of evolutionary trees for the microsoft windows environment. Comput. Appl. Biosci. 10, 569-570.

Van de Peer, Y. and De Wachter, R. (1997) Construction of evolutionary distance trees with TREECON for Windows: accounting for variation in nucleotide substitution rate among sites. Comput. Appl. Biosci. 13, 227-230.

Van der Wielen, P. W. J. J., Rovers, G. M. L. L., Scheepens, J. M. A. and Biesterveld, S. (2002) *Clostridium lactatifermentans* sp. nov., a lactate-fermenting anaerobe isolated from the caeca of a chicken. Int. J. Syst. Evol. Microbiol. 52, 921-925.

Van Dyke, M. I. and McCarthy, A. J. (2002) Molecular biological detection and characterization of *Clostridium* populations in municipal landfill sites. Appl. Environ. Microbiol. 68, 2049-2053.

Vangylswyk, N. O. and Vandertoorn, J. J. T. K. (1985) *Eubacterium uniforme* sp. nov. and *Eubacterium xylanophilum* sp. nov., fiber-digesting bacteria from the rumina of sheep fed corn stover. Int. J. Syst. Bact.. 35, 323-326.

Vangylswyk, N. O. and Vandertoorn, J. J. T. K. (1986) Description and designation of a neotype strain of *Eubacterium cellulosolvens* (*Cillobacterium cellulosolvens* bryant, small, bouma and robinson) holdeman and moore. Int. J. Syst. Bact. 36, 275-277.

Vangylswyk, N. O. and Vandertoorn, J. J. T. K. (1987) *Clostridium aerotolerans* sp. nov., a xylanolytic bacterium from corn stover and from the rumina of sheep fed corn stover. Int. J. Syst. Bact. 37, 102-105.

Vanlier, J. B., Grolle, K. C. F., Frijters, C. T. M. J., Stams, A. J. M. and Lettinga, G. (1993) Effects of acetate, propionate, and butyrate on the thermophilic anaerobic degradation of propionate by methanogenic sludge and defined cultures. Appl. Environ. Microbiol. 59, 1003-1011.

Voolapalli, R. K. and Stuckey, D. C. (1999) Relative importance of trophic group concentrations during anaerobic degradation of volatile fatty acids. Appl. Environ. Microbiol. 65, 5009-5016.

Wang, M., Ahrne, S., Antonsson, M. and Molin, G. (2004) T-RFLP combined with principal component analysis and 16S rRNA gene sequencing: An effective strategy for comparison of fecal microbiota in infants of different ages. J. Microbiol. Meth. 59, 53-69.

Wang, X., Heazlewood, S. P., Krause, D. O. and Florin, T. H. J. (2003) Molecular characterization of the microbial species that colonize human ileal and colonic mucosa by using 16S rDNA sequence analysis. J. Appl. Microbiol. 95, 508-520.

Wang, Y., Hsieh, Y. P., Landing, W. M., Choi, Y. H., Salters, V. and Campbell, D. (2002) Chemical and carbon isotopic evidence for the source and fate of dissolved organic matter in the northern Everglades. Biogeochemistry 61, 269-289.

Warikoo, V., McInerney, M. J., Robinson, J. A. and Suflita, J. M. (1996) Interspecies acetate transfer influences the extent of anaerobic benzoate degradation by syntrophic consortia. Appl. Environ. Microbiol. 62, 26-32.

Warnick, T. A., Methe, B. A. and Leschine, S. B. (2002) *Clostridium phytofermentans* sp. nov., a cellulolytic mesophile from forest soil. Int. J. Syst. Evol. Microbiol. 52, 1155-1160.

Weber, S., Stubner, S. and Conrad, R. (2001) Bacterial populations colonizing and degrading rice straw in anoxic paddy soil. Appl. Environ. Microbiol. 67, 1318-1327.

Winter, J., Popoff, M. R., Grimont, P. and Bokkenheuser, V. D. (1991) *Clostridium orbiscindens* sp. nov., a human intestinal bacterium capable of cleaving the flavonoid c-ring. Int. J. Syst. Bact. 41, 355-357.

Wright, A. L. and Reddy, K. R. (2001a) Heterotrophic microbial activity in northern Everglades wetland soils. Soil Sci. Soc. Am. J. 65, 1856-1864.

Wright, A. L. and Reddy, K. R. (2001b) Phosphorus loading effects on extracellular enzyme activity in Everglades wetland soils. Soil Sci. Soc. Am. J. 65, 588-595.

Wu, M., Song, L. S., Ren, J. P., Kan, J. J. and Qian, P. Y. (2004) Assessment of microbial dynamics in the Pearl River Estuary by 16S rRNA terminal restriction fragment analysis. Cont. Shelf Res. 24, 1925-1934.

Wu, Y. G., Sklar, F. H. and Rutchey, K. (1997) Analysis and simulations of fragmentation patterns in the Everglades. Ecol. Appl. 7, 268-276.

Yanling, H., Ding, Y. F. and Long, Y. Q. (1991) Two cellulolytic *Clostridium* species: *Clostridium cellulosi* sp. nov. and *Clostridium cellulofermentans* sp. nov. Int. J. Syst. Bact. 41, 306-309.

BIOGRAPHICAL SKETCH

Ilker Uz was born in Afyon, Turkey, on the 7th of January, 1972. In 1993, he graduated with a B.Sc. in soil science from Ankara University, Ankara, Turkey, at the top of his graduating class. During his undergraduate training he decided to pursue his studies further and become an academician. Therefore, immediately after graduation, he entered the exam for graduate work at the Soil Science Department at the Faculty of Agriculture of Akdeniz University. He was offered a research and teaching assistantship by the department, which in Turkey is considered to be the first step toward academic life. In June 1995, he was selected to receive the Turkish Ministry of National Education scholarship to pursue his graduate studies in the United States. In January 1997, he started his graduate study under the supervision of Dr. Andrew Ogram in the Department of Soil and Water Science at the University of Florida. He was awarded the degree of Master of Science in December 1998. He started his Ph.D. studies in the same research team in January 1999. His Ph.D. project focused on microbial ecology of the Florida Everglades. He was also involved in various projects such as the landfill project. Upon completion of his Ph.D., he plans to continue to improve his experience in science and become a faculty member in a university.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Andrew V. Ogram, Chair
Associate Professor of Soil and Water Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Konda R. Reddy
Graduate Research Professor of Soil and Water Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Donald A. Graetz
Professor of Soil and Water Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Joseph P. Prenger
Research Assistant Professor of Soil and Water Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Kenneth M. Portier
Associate Professor of Statistics

This dissertation was submitted to the Graduate Faculty of the College of Agricultural and Life Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 2005

Dean, College of Agricultural and Life Sciences

Dean, Graduate School